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*No. 1. SIMON FLEXNER, J. W. JOBLING, and MAUD L. MENTEN. Experimental studies on tumor. Plates 1–28. (Issued June 30, 1910.) Price, $1.00.

*Out of print.

THE COMBINATION OF GELATIN WITH HYDROCHLORIC ACID.

II. New Determinations of the Isoelectric Point and Combining Capacity of a Purified Gelatin.

By DAVID I. HITCHCOCK.

(From the Department of Physiology, Yale University, New Haven.)

(Accepted for publication, December 19, 1928.)

I.

Introduction.

About 6 years ago the writer[1] reported the results of hydrogen electrode titrations and conductivity titrations of gelatin with hydrochloric acid. These experiments led to the conclusion that 1 gm. of gelatin was capable of combining with 8.6 to 8.9 $\times 10^{-4}$ equivalents of HCl. The latter figure appeared to represent a maximum which was not exceeded even in the presence of a large excess of acid. In this earlier work the experimental data were corrected on the assumption that gelatin was isoelectric and combined with no measurable acid at pH 4.70. Hence the validity of the figure for the combining capacity is limited by this assumption, as well as by the fact that it was obtained only with gelatin obtained from one particular source (Peter Cooper's Glue Factory) and purified in one particular way (Loeb's method of washing).

The difference between purified gelatins of different origin was brought out by the work of Kraemer and Dexter[2] in their study of the effect of pH on the light-scattering capacity of gelatin sols and gels. They assumed the pH where this effect was at a maximum to be

[1] Hitchcock, D. I., (*a*) *J. Gen. Physiol.*, 1921–22, iv, 733; (*b*) *J. Gen. Physiol.*, 1923–24, vi, 95; (*c*) *J. Gen. Physiol.*, 1922–23, v, 383; (*d*) *J. Gen. Physiol.*, 1923–24, vi, 201.

[2] Kraemer, E. O., and Dexter, S. T., *J. Phys. Chem.*, 1927, xxxi, 764.

that of the isoelectric point, and found this pH to be close to 5 for hide gelatins, 5.5 for an ossein gelatin, and 8 for a pigskin gelatin. They concluded that previous estimates of the isoelectric point of hide gelatins were too low, and showed that many of the data of previous workers were not inconsistent with an isoelectric point at pH 5.

The combining capacity of "Difco" gelatin, purified by Loeb's method, was found by Chapman, Greenberg, and Schmidt[3] to be 10.4×10^{-4} equivalents of acid dye per gram of gelatin. They suggested that the writer's lower figures were due to the fact that the second basic groups of lysine, arginine and histidine were only 80 to 90 per cent dissociated (combined with H^+) in HCl even at pH 1. If this is true for these groups in gelatin, the curve of combined H^+ in HCl should not be horizontal between pH 2 and 1, but still ascending.

The present paper records an attempt to locate more exactly the isoelectric point of a gelatin from the same source as that used in the earlier experiments, to determine whether or not its combination curve with hydrogen ion is horizontal between pH 2 and 1, and to determine as exactly as possible the extent of its combining capacity for each of the ions of hydrochloric acid. The latter determinations have been made by electromotive force measurements of a cell without liquid junction, of the type used in exact thermodynamic studies in inorganic chemistry, but not previously used, so far as the writer is aware, in studies of protein solutions.

II.

Isoelectric Point.

The gelatin used in this work was part of a single 5 pound package of Cooper's gelatin, purchased from Peter Cooper's Glue Factory, Gowanda, N. Y. It was purified in 50 gm. lots by Loeb's method of washing, as improved by Northrop and Kunitz.[4] It was dried by alcohol and ether in the air. Solutions containing about 13 per cent gelatin by weight were prepared by dissolving 50 gm. of this air-dry preparation in 250 cc. of distilled water. The exact concentration of each stock solution was obtained by weighing the solution delivered at 40° by a 10 cc.

[3] Chapman, L. M., Greenberg, D. M., and Schmidt, C. L. A., *J. Biol. Chem.*, 1927, lxxii, 707.

[4] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1927–28, xi, 477.

pipette, and evaporating to dryness another sample delivered by the same pipette under the same conditions. Dry weights were considered constant when they decreased less than 0.2 per cent per day, which was the case after 5 to 15 days at 110°C. This gelatin was analyzed for total nitrogen by the Kjeldahl method, and found to contain 18.0 per cent N on a dry weight basis. Its ash content was found to be 0.04 per cent, which may be compared with the value 0.1 per cent obtained[5] for gelatin purified by Loeb's original method. Its freedom from electrolytes may also be inferred from the conductivity data given in Table I.

The isoelectric point of the gelatin used in this work was determined by two methods, the pH of minimum osmotic pressure and the pH of

TABLE I.

Specific Conductivity at 30° of Purified Gelatin in Distilled Water.

Concentration of gelatin, gm. per 1000 gm. H_2O	Specific conductivity, $10^6 \times$ reciprocal ohms		
	Solution	Water	Difference
52.1	2.98*	0.12*	2.86
89.3	3.94*	0.12*	3.82
156.0	5.59†	0.58†	5.01

* Twice distilled water.
† Once distilled water.

maximum turbidity. Osmotic pressure measurements were made at 30°C., essentially as described by Loeb,[6] using 1 per cent and 2 per cent gelatin solutions whose pH was varied by very small additions of HCl or NaOH. The differences in level of the inside and outside solutions were measured to the nearest millimeter after 1 or 2 days. Each reading was corrected by subtracting the capillary rise of water in the tubes used, about 6 mm. The concentration of gelatin inside each membrane was determined by dry weight determinations, and the results were brought to a common basis by dividing the corrected height in millimeters of solution by the concentration of gelatin in grams per kilo of water. The pH values were obtained with bubbling hydrogen electrodes at 30°C., and are based on Scatchard's value[7]

[5] Loeb, J., *J. Am. Chem. Soc.*, 1922, xliv, 213.

[6] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1st edition, 1922; 2nd edition, 1924.

[7] Scatchard, G., *J. Am. Chem. Soc.*, 1925, xlvii, 696.

of 0.841 for the activity coefficient of H^+ in 0.1 M HCl (pH = 1.075). This standard is 0.04 pH higher than that previously used by the writer. Liquid contact was made by bridges of saturated KCl in agar-agar, and liquid junction potentials were assumed constant.

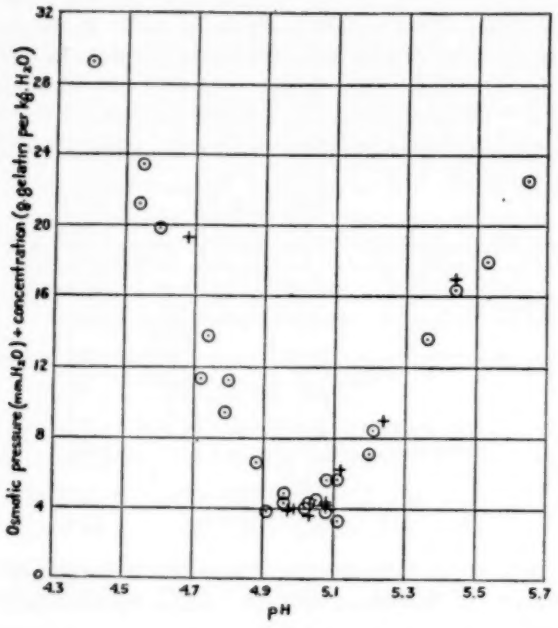

FIG. 1. Effect of pH on osmotic pressure of gelatin solutions near the isoelectric point. Ordinates are osmotic pressures in millimeters of water divided by concentrations in grams gelatin per kilo of water. Abscissæ are pH values. Circles represent experiments with about 10 gm. gelatin per 1000 gm. H_2O; crosses represent experiments at twice this concentration. The points indicate that the minimum osmotic pressure, and hence the isoelectric point of this gelatin, was located at pH 5.02 $\pm$ 0.05.

The pH values obtained with these agar bridges differed by less than 0.01 pH from those obtained with a liquid junction made around a ground glass stopper.

The results of six osmotic pressure experiments are plotted against pH in Fig. 1. The points show no minimum in the vicinity of pH

4.7, but indicate that this sample of gelatin was isoelectric at pH 5.02 ±0.05.

Confirmation of this location of the isoelectric point of the gelatin used was obtained from observations of the effect of pH on the opacity of gels. The pH was varied by small additions of HCl or NaOH, and measured, as described above, at 30°. No attempt was made to get quantitative photometric measurements of the scattered light, but the gel of maximum turbidity was located by inspection of 10 cc. samples in test-tubes after standing overnight in a refrigerator at about 8°C. The results are given in Table II. Evidently for this gelatin the pH of maximum opacity is 5.05 ±0.05. It appears to be independent of the concentration of the gelatin, and to differ very little from the pH

TABLE II.

Effect of pH on the Opacity of Gelatin Gels.

Concentration of gelatin, gm. per 1000 gm. H_2O	Composition of solvent	pH of maximum opacity	pH of next samples in series
2	0.00005 N HCl	5.05	4.95, 5.11*
10	0.0001 N HCl	5.03	4.93, 5.06*
20	H_2O	5.07*	4.99, 5.17

* pH of gelatin + water.

of the gelatin in water, 5.08 ±0.03, which also seems to vary in no regular way with the concentration. Hence the isoelectric point of this gelatin may be taken as pH 5.05 ±0.05, and any correction for acid or base contained in the purified gelatin is so small as to be safely neglected in the calculations of combining capacity reported below.

III.

Combining Capacity for Hydrogen Ion.

The curves previously obtained by the writer[1a,e] for the amount of hydrogen ion bound by gelatin as a function of pH are open to criticism because of the scattering of the points between pH 1 and 2. This scattering was due to the fact that, for 1 per cent solutions, the quantity plotted was a small difference between two larger concentrations of acid, each of which might be affected by the error inherent in a pH

determination. Hence the percentage error in the calculated amount of bound acid was often unreasonably large. It seems likely that such errors may account for some of the peculiar combination curves of protein with acid obtained by others.[8] In the present work it has proved possible to reduce this error by using higher concentrations of gelatin.

The solutions were made up by weight from hydrochloric acid solutions standardized by Na_2CO_3 and AgCl, and gelatin solutions standardized by dry weight determinations at 110°C. The pH determinations were made in wide test-tubes, using platinized wire electrodes and bubbling hydrogen. Contact was made with a saturated KCl calomel electrode through bridges of saturated KCl in 3 per cent agar-agar jelly. The bridge tubes were drawn out and bent up at the end to minimize diffusion of KCl into the solutions. The bridges were dipped into the solutions only while readings were being taken. The E. M. F. of the cells was measured to 0.1 millivolt with a Leeds and Northrup Type K potentiometer. In some cases 2 or 4 hydrogen electrodes were used with samples of the same solution in different tubes; in other cases a single electrode was used with a given solution, but the cell was refilled and the measurement repeated. Readings were considered constant when they checked within 0.5 millivolt (often less) and showed no drift over a period of 20 or 30 minutes. The E.M.F. readings were corrected to 1 atmosphere of hydrogen by the tables of Clark.[9] The cells were kept at 30° ±0.02 by an electrically controlled water bath.

The pH values were based on the standard mentioned above. Repeated measurements with a single calomel electrode (not made with especially purified materials) against hydrogen electrodes in 0.1 M HCl gave the E_0 value for this cell at 30° as 0.2405, and the pH values were obtained from the Nernst formula in the form

$$\text{pH} = \frac{E - 0.2405}{0.06015}.$$

[8] Lloyd, D. J., and Mayes, C., *Proc. Roy. Soc. London, Series B*, 1922, xciii, 69. Hoffman, W. A., and Gortner, R. A., in Holmes, H. N., Colloid symposium monograph, New York, 1925, ii, 209.

[9] Clark, W. M., The determination of hydrogen ions, Baltimore. 2nd edition, 1922.

TABLE III.

Determination of H^+ Combined with Gelatin from Hydrogen Electrode Measurements with KCl-agar Junction.

m	g	E	pH	a_H	γ_H	m_H	b_H	$\frac{b_H}{g} \times 10^4$
0	2	0.5470	5.11	7.8×10^{-6}	1.000	7.8×10^{-6}	0	0
0	10	0.5445	5.06	8.7×10^{-6}	1.000	8.7×10^{-6}	0	0
0	20	0.5455	5.07	8.5×10^{-6}	1.000	8.5×10^{-6}	0	0
0	40	0.5445	5.06	8.7×10^{-6}	1.000	8.7×10^{-6}	0	0
0.01029	31.3	0.4850	4.07	8.6×10^{-5}	0.921	9.3×10^{-5}	0.01020	3.26 ± 0.00
0.0550	90.0	0.4525	3.52	3.0×10^{-4}	0.860	3.5×10^{-4}	0.0546	6.07 ± 0.00
0.02058	31.4	0.4425	3.36	4.4×10^{-4}	0.895	4.9×10^{-4}	0.02009	6.40 ± 0.00
0.0543	70.4	0.4270	3.10	7.9×10^{-4}	0.861	9.2×10^{-4}	0.0534	7.58 ± 0.00
0.0844	90.2	0.3910	2.50	0.0032	0.846	0.0037	0.0807	8.95 ± 0.00
0.0531	51.8	0.3790	2.30	0.0050	0.861	0.0058	0.0473	9.13 ± 0.02
0.0830	70.1	0.3510	1.84	0.0146	0.847	0.0172	0.0658	9.39 ± 0.04
0.0521	34.2	0.3465	1.76	0.0174	0.852	0.0204	0.0317	9.27 ± 0.15
0.1131	89.5	0.3375	1.61	0.0245	0.839	0.0292	0.0839	9.37 ± 0.07
0.0816	51.6	0.3335	1.55	0.0282	0.847	0.0333	0.0483	9.36 ± 0.16
0.1052	71.7	0.3300	1.49	0.0324	0.841	0.0385	0.0667	9.30 ± 0.13
0.1110	70.3	0.3255	1.41	0.0389	0.840	0.0463	0.0647	9.20 ± 0.16
0.0795	34.7	0.3245	1.40	0.0398	0.848	0.0470	0.0325	9.37 ± 0.32
0.1046	56.1	0.3225	1.36	0.0437	0.841	0.0519	0.0527	9.39 ± 0.22
0.1087	51.6	0.3190	1.30	0.0501	0.840	0.0597	0.0490	9.51 ± 0.26
0.1035	41.2	0.3160	1.25	0.0562	0.841	0.0668	0.0367	8.91 ± 0.36
0.1062	34.7	0.3135	1.21	0.0617	0.840	0.0735	0.0327	9.43 ± 0.46
0.1019	27.3	0.3120	1.19	0.0646	0.841	0.0768	0.0251	9.20 ± 0.65
0.2310	121.2	0.3005	1.00	0.1000	0.836	0.1196	0.1114	9.19 ± 0.23
0.2192	73.0	0.2935	0.88	0.1318	0.836	0.1576	0.0616	8.44 ± 0.49

m = molality of HCl.

g = gm. gelatin per kilo H_2O.

E = observed E.M.F. in volts, corrected to 1 atm. H_2.

$$\text{pH} = \frac{E - 0.2405}{0.06015}.$$

a_H = activity of H^+ = antilog $(-\text{pH})$.

γ_H = activity coefficient of H^+, from Scatchard's data.

$m_H = \frac{a_H}{\gamma_H}$ = molality of free H^+.

b_H = molality of combined $H^+ = m - m_H$.

$\frac{b_H}{g} \times 10^4$ = mols of H^+ combined with 10,000 gm. gelatin.

The numbers following the $\pm$ sign represent the effect of an error of $\pm$ 0.5 millivolt in E.

The amounts of combined hydrogen ion were calculated by the method used by Cohn,[10] which differs slightly from that used by Loeb[6] and by the writer[1] in previous work. If b_H = molality of bound H^+, m = total molality of HCl, and a_H = activity of H^+ as given by pH measurements, then

$$b_H = m - \frac{a_H}{\gamma_H}.$$

Here γ_H was taken as the activity coefficient of H^+, according to Scatchard[7], in a pure HCl solution of molality m. This is equivalent to assuming the ionic strength principle of Lewis and Randall[11] and assigning to the gelatin-H^+ complex an effective valence of one. In the method of calculation previously used the terms m and b_H represented volume normal concentrations and γ_H was taken as the activity coefficient of H^+ in that concentration of pure acid having a measured hydrogen ion activity equal to that found for the gelatin-HCl solution. This was equivalent to assuming that the gelatin chloride present had no effect on the activity of H^+ in the excess HCl. The present assumption seems more reasonable than that formerly used because of the results with acid-salt mixtures cited by Lewis and Randall.[11]

The values of b_H were divided by the concentrations of gelatin to get the number of equivalents of H^+ combined with unit weight of gelatin. This quantity is given in the last column of Table III, and is plotted against pH in Fig. 2. The curve in Fig. 2 was drawn to represent the best smooth relation between the experimental points. It will be noted that the points do not show any evidence of a further upward trend with decrease of pH from 2 to 1, as would be expected if the explanation proposed by Schmidt[3] and his co-workers were correct. Except for two points which lie below the curve, the points lie fully as close to a horizontal line between pH 2 and 1 as could be expected from the effect of a possible error of 0.5 millivolt in the E.M.F. of the cell. Although in many cases the agreement of duplicates was

[10] Cohn, E. J., *Physiol. Rev.*, 1925, v, 349.

[11] Lewis, G. N., and Randall, M., *J. Am. Chem. Soc.*, 1921, xliii, 1112; Thermodynamics and the free energy of chemical substances, New York and London, 1923.

better than this, the measurements with HCl alone differed so much that the precision is probably not better than ±0.5 millivolt or ±0.01 pH for these agar bridge measurements.

The experiments thus confirm the writer's earlier conclusion that the amount of H^+ bound by 1 gm. of gelatin is constant between pH 2 and 1. For this gelatin this quantity is 9.35×10^{-4} equivalents of H^+ per gram of gelatin. This figure was confirmed by conductivity titrations of gelatin with HCl, carried out by the method previously

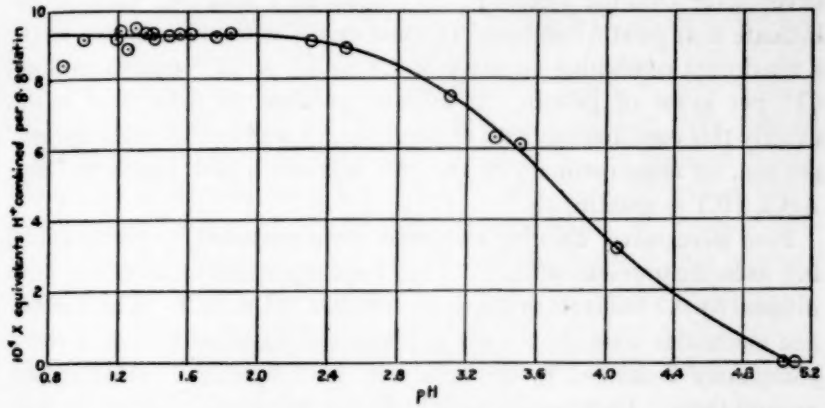

FIG. 2. The combination of gelatin with hydrogen ion from HCl. Ordinates are equivalents of H^+ combined with 1 gm. of gelatin, multiplied by 10^4, as calculated from hydrogen electrode measurements with an agar-KCl junction. Abscissæ are pH values of the gelatin-HCl solutions, obtained from the same measurements. The points indicate a maximum of 9.35×10^{-4} equivalents of H^+ combined with 1 gm. of gelatin between pH 2 and 1.

described.[1, d] The end-points of three titrations, using different concentrations of gelatin, gave the following results: 9.39, 9.37, 9.47 $\times 10^{-4}$; average, 9.41×10^{-4} equivalents per gram.

The difference between these values and those obtained earlier (8.9 by hydrogen electrode, 8.6 by conductivity) is more than accounted for by the different values assigned to the isoelectric point. Such differences might well be due to the very doubtful uniformity of the original commercial gelatin. It should be possible to avoid such differences and uncertainties by the use of gelatin prepared from definite materials in a definite way. A committee of the Leather and

Gelatin Division of the American Chemical Society has proposed tentative specifications for a standard gelatin for scientific purposes, and the writer hopes to be able to report later on the acid-combining capacity of gelatin prepared in accordance with these specifications.

IV.

Combination with Hydrogen and Chloride Ions.

A recalculation of old data[1,e] and a few new experiments with silver-silver chloride electrodes in gelatin-HCl solutions seemed to indicate that gelatin combined to some extent with chloride ion, with a maximum combining capacity of 1.5 to 2.5 $\times$ 10^{-4} equivalents of Cl^- per gram of gelatin. It seemed possible to determine more exactly this combination with chloride ion, as well as that with hydrogen ion, by measurements of the cell without liquid junction: Ag, AgCl, HCl + gelatin, H_2.

Four silver-silver chloride electrodes were prepared by electroplating, as in the previous work.[1,e] They were replated whenever any two differed by 0.2 millivolt in the same solution, 0.1 M HCl. The hydrogen electrodes were short coils of platinized platinum wire, and were completely immersed in the solutions, with hydrogen bubbling up around them. Commercial tank hydrogen was used. Its purity was tested by hydrogen electrode measurements in 0.1 M HCl with hydrogen passed through alkaline permanganate, alkaline pyrogallate, and distilled water. Identical readings were obtained with only the washing in water at 30°; accordingly the rest of the purifying train was omitted in the experiments. The vessels used were simple U-tubes about 2 cm. wide in the vertical arms and 1 cm. at the bend. A few measurements were made with the AgCl and H_2 electrodes in a single test-tube, but the E.M.F. so obtained decreased with time. The U-tubes, by protecting the AgCl from H_2, gave readings which were constant to 0.2 millivolt for several hours. In each experiment 4 cells were set up with the same solution, and readings taken to the nearest 0.05 millivolt for 3 to 5 hours. The E.M.F. values were plotted against time and an average value selected from those points which could best be represented by a horizontal line over a period of at least 1 hour. The values so obtained are probably reliable to 0.1

millivolt or better as a measure of the true E.M.F. in any one experiment. Many experiments had to be discarded because of lack of constancy and reproducibility in the E.M.F. It was found in some cases that the erratic behavior was due to toluene which had been used to protect the stock solution of gelatin. In the course of repeated meltings and settings of the gelatin, the toluene seemed to become permanently emulsified. Other solutions which had been in contact with thymol gave low and rapidly drifting E.M.F. values, an effect which was reproduced with HCl containing thymol. The data reported below were obtained with gelatin to which no preservative was added. The readings were obtained with AgCl electrodes which had not been freshly plated for 2 or 3 months, but had been kept in 0.1 M HCl. After each day's use in a gelatin solution they were washed with hot water, distilled water, and 0.1 M HCl. At the end of this series of experiments the E.M.F. obtained with these electrodes in the cell Ag, AgCl, HCl (0.1 M), H_2 was just as constant and reproducible as at the start of the series, but was 0.6 millivolt lower. This lowering was either a slow time effect or else an effect of the contact with gelatin. The calculations were based on this final HCl reading as a standard rather than on the initial reading, although the latter was again reproduced on replating the electrodes.

The data and calculations are given in Table IV. Each figure is the mean of two experiments, each done with four sets of electrodes. The E.M.F. of the cell Ag, AgCl, HCl, H_2 at 30°C. is given by the thermodynamic equation

$$E = E_0 - 0.06015 \log a_H \, a_{Cl} \qquad (1)$$

in which a_H and a_{Cl} represent the activities of the ions and E_0 is the E.M.F. of a hypothetical cell in which the product $a_H \, a_{Cl}$ is equal to one. The determination of E_0 by extrapolation has been discussed elsewhere.[12] For the present purpose it is not necessary to know the absolute value of this quantity. Equation (1) may be written

$$E = E_0 - 0.1203 \log \gamma - 0.06015 \log m_H \, m_{Cl} \qquad (2)$$

where γ is the geometrical mean activity coefficient of the two ions and m_H and m_{Cl} are their respective molalities. This form of equation

[12] Hitchcock, D. I., *J. Am. Chem. Soc.*, 1928, l, 2076.

has been applied to the calculation of the activity coefficient of HCl in the presence of other chlorides. The results of Harned,[13] as quoted by Lewis and Randall,[11] show that in mixtures of HCl with LiCl, NaCl,

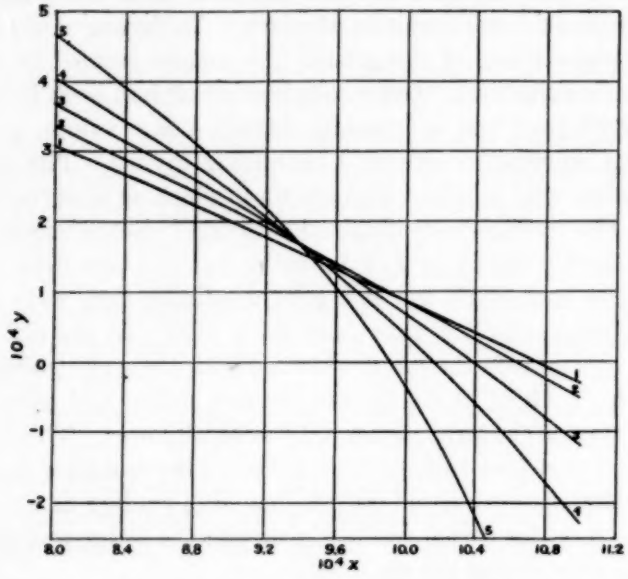

FIG. 3. Graphical determination of the combining capacity of gelatin with H^+ and Cl^- from E.M.F. measurenents of the cell Ag, AgCl, HCl + gelatin, H_2, without liquid junction. Each experiment leads to an equation containing two unknowns, x and y, the equivalents of H^+ and Cl^- combined with 1 gm. of gelatin. In the figure the abscissæ are values of $10^4\,x$ and the ordinates are $10^4\,y$. Each curve represents the relation between these variables for one experiment, and is marked with the number of the experiment. The true values of these quantities, if constant for all the experiments, should be given by a single intersection of all the curves. The figure shows that four of the five curves intersect at the point where $x = 9.4 \times 10^{-4}$ and $y = 1.7 \times 10^{-4}$.

or KCl, at a constant total molality of 0.1 or 0.2, the activity coefficient is the same as that in pure HCl of the same total molality. The writer has confirmed this by experiments with 0.1 M HCl partly neutralized by NaOH, KOH, or NH_4OH. Accordingly in the calcula-

[13] Harned, H. S., *J. Am. Chem. Soc.*, 1920, xlii, 1808.

tion of the gelatin experiments it will be assumed that the same principle applies; in other words, the partial neutralization of dilute HCl by gelatin is assumed not to affect the mean activity coefficient of its ions. On this basis the expression $E_0 - 0.1203 \log \gamma$ is assumed constant and equal to the value obtained without gelatin. An experiment done at the conclusion of the gelatin series with 0.1008 M HCl gave E =

TABLE IV.

Combining Capacities of Gelatin for H^+ and Cl^- from E.M.F. of Cells without Liquid Junction.

Experiment No.	m	g	E (observed)	$\frac{E - 0.2305}{0.06015}$	$m_H\, m_{Cl}$	E (calculated)
1	0.1005	13.5	0.3546	2.063	0.00865	0.3547
2	0.1014	27.3	0.3589	2.135	0.00733	0.3589
3	0.1025	44.5	0.3652	2.239	0.00577	0.3652
4	0.1036	59.8	0.3721	2.354	0.00443	0.3721
5	0.1048	77.4	0.3827	2.530	0.00295	0.3828

m = molality of HCl.

g = concentration of gelatin in gm. per kilo H_2O.

E (observed) = E.M.F. in volts of cell Ag, AgCl, HCl + gelatin, H_2 (1 atm.).

0.2305 = $E_0 - 0.1203 \log \gamma$, from E (observed) = 0.3504 for 0.1008 M HCl without gelatin.

$m_H\, m_{Cl}$ = antilog $\frac{0.2305 - E}{0.06015}$ = product of molalities of ions.

E (calculated) = $0.2305 - 0.06015 \log (m - gx)(m - gy)$ where m and g have values in columns 2 and 3, $x = 9.4 \times 10^{-4}$, and $y = 1.7 \times 10^{-4}$. The latter values were obtained graphically in Fig. 3 from the values of $m_H\, m_{Cl}$ in column 6 and the relation $m_H\, m_{Cl} = (m - gx)(m - gy)$.

0.3504. From this it follows that $E_0 - 0.1203 \log \gamma$, which is equal to $E + 0.1203 \log m$, is 0.2305. Now equation (2) may be rewritten

$$\frac{E - 0.2305}{0.06015} = -\log (m - g\,x)(m - g\,y) \qquad (3)$$

where x and y are numbers of equivalents of H^+ and Cl^- combined with 1 gm. of gelatin, and g is the concentration of the gelatin in grams per kilo H_2O. For each experiment E, m, and g were measured; hence the equation contains the two unknowns x and y. The concentrations in the experiments were such that x and y might be expected to be constant,

the gelatin being combined to the maximum possible extent with both H^+ and Cl^-. This constancy may be tested, and the equation solved for both x and y, by plotting y as a function of x for each experiment. This was done by using the observed values of E, m, and g, assuming values for x, and calculating y. If the values of x and y are really constant, all the curves should intersect in a single point whose coordinates are the true values of x and y. The curves in Fig. 3 were obtained from the data of Table IV in this way, and the validity of the assumed constancy of x and y is justified by the single intersection of four curves. The form of equation (3) is such that algebraically x and y are interchangeable. The experiments in the preceding part of this paper indicate that the higher value belongs to x, the equivalents of H^+ combined with 1 gm. of gelatin. The experiments therefore lead to the conclusion that 1 gm. of gelatin in 0.1 M HCl combines with a maximum of 9.4×10^{-4} equivalents of H^+ and 1.7×10^{-4} equivalents of Cl^-. The consistency of these figures with the experimental data is shown by the last column in Table IV, which gives the E.M.F. calculated by equation (3), using the observed values of m and g and the above values of x and y. The agreement of these with the observed values shows that the failure of Curve 1 in Fig. 3 to intersect the others at a single point is not due to an unreasonable experimental error in the E.M.F., but simply due to the magnification of error inherent in an experiment with a low protein concentration. These figures are probably more reliable than those obtained from the agar bridge data because of the absence of liquid junction errors in these cells. It is hoped that this method may be used later in a study of the standard gelatin mentioned above, as well as with other proteins.

V.

Summary.

1. Cooper's gelatin purified according to Northrop and Kunitz exhibited a minimum of osmotic pressure and a maximum of opacity at pH 5.05 ±0.05. The pH of solutions of this gelatin in water was also close to this value. It is inferred that such gelatin is isoelectric at this pH and not at pH 4.70.

2. Hydrogen electrode measurements with KCl-agar junctions were

made with concentrated solutions of this gelatin in HCl up to 0.1 M. The combination curve calculated from these data is quite exactly horizontal between pH 2 and 1, indicating that 1 gm. of this gelatin can combine with a maximum of 9.35×10^{-4} equivalents of H^+.

3. Conductivity titrations of this gelatin with HCl gave an end-point at $9.41\ (\pm 0.05) \times 10^{-4}$ equivalents of HCl per gram gelatin.

4. E.M.F. measurements of the cell without liquid junction, Ag, AgCl, HCl + gelatin, H_2, lead to the conclusion that this gelatin in 0.1 M HCl combines with a maximum of 9.4×10^{-4} equivalents of H^+ and 1.7×10^{-4} equivalents of Cl^- per gram gelatin.

The writer wishes to ackowledge the faithful assistance of Miss Esther R. Mason, who did most of the experimental work reported in this paper.

CHEMICAL ANTAGONISM OF IONS.

III. Effect of Salt Mixtures on Gelatin Activity.

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I

INTRODUCTION.

Two previous papers[1] showed that the anomalous effect of Mg^{++} ions[2] on the activity of oxalate diions ($C_2O_4^{=}$) could be counteracted by the addition of salts of Na^+ or K^+. A similar antagonism was found between $SO_4^{=}$ and Cl^-, as well as between Mg^{++} and $SO_4^{=}$ in their effect on the oxalate diion. No antagonism was found between Na^+ and K^+.

In this paper we will extend our observations to the effect of single salts and of salt mixtures upon the ionic activity of gelatin.[3] It will be shown that, of the three ions Na^+, K^+ and Mg^{++}, each has its individual effect when present alone with gelatin; and that the effect of a mixture of two or more of these cations is quite different from the effect of one alone.

The experimental data were obtained in the pH range from 6.7 to 7.6 in order to be near the physiological range, the pH of the solutions without salt being 7.367. An accuracy reproducible to 0.001 to 0.003 pH was obtained, although with difficulty.

The ionizable groups of gelatin[4] are given below together with their degree of ionization at pH 7.367, which was the pH of the gelatin solution without salts in our experiments.

[1] Simms, H. S., *J. Gen. Physiol.*, 1928, xii, 241, 259.
[2] Simms, H. S., *J. Phys. Chem.*, 1928, xxxii, 1121, 1495.
[3] Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 613.
[4] Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 629.

Acid groups (ionized at pH's higher than pG')	pG' values[5]	100 α = per cent of ionization	Basic groups (ionized at pH's lower than pG')	pG' values[5]	100 α = per cent of ionization
Aspartic and glutamic acids (R — COOH)	3.5	100	Prearginine[4]	4.6	0
			Histidine	6.1	5
			Arginine	8.1	84
(Tyrosine RΦ — OH)	9.4	1	Lysine	10.6	100

Arginine is the only group having appreciable amounts of both the ionized and the non-ionized form at this pH (84 and 16 per cent respectively).

To express this in a different way: the change in pH of the gelatin solutions due to addition of salt is an indication of a change in the ionic activity of one or more of the ionic species present. There are only two ionic species of the gelatin present in appreciable quantities;[6] one in which the arginine group is ionized, the other in which it is non-ionized. The other groups remain essentially the same.

The same statements should apply to any other protein in this range and our results should be typical of proteins in general. At present we have not made observations in other pH ranges.

II.

RESULTS.

A. Solutions Containing Na^+ and K^+ Ions.

The effect of Na^+ (as NaCl) on Na gelatinate may be seen in Figs. 1 and 2A. All the solutions of Na gelatinate contained 1.25 per cent gelatin to which enough NaOH has been added to bring the pH to 7.367 without addition of salt. The pH of this solution is seen to decrease very rapidly with small additions of NaCl; the influence of

[5] The pG' values (titration indices) represent the mid-points of the buffer ranges due to the respective groups. Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

[6] We neglect the histidine group which is only 5 per cent ionized. That this is permissible is shown by the data on K gelatinate with a salt-free pH of 7.203 where the histidine group is 7 per cent ionized and the arginine group is 89 per cent ionized.

further additions becomes less and less as the concentration of salt increases—but the effect up to above 1.0 molar is to *decrease the pH*.

KCl on the other hand *first decreases* the pH until about 0.01 molar K^+ ion is present. The curve then reverses and further additions of KCl *increase* the pH. This is true whether the KCl is added to Na

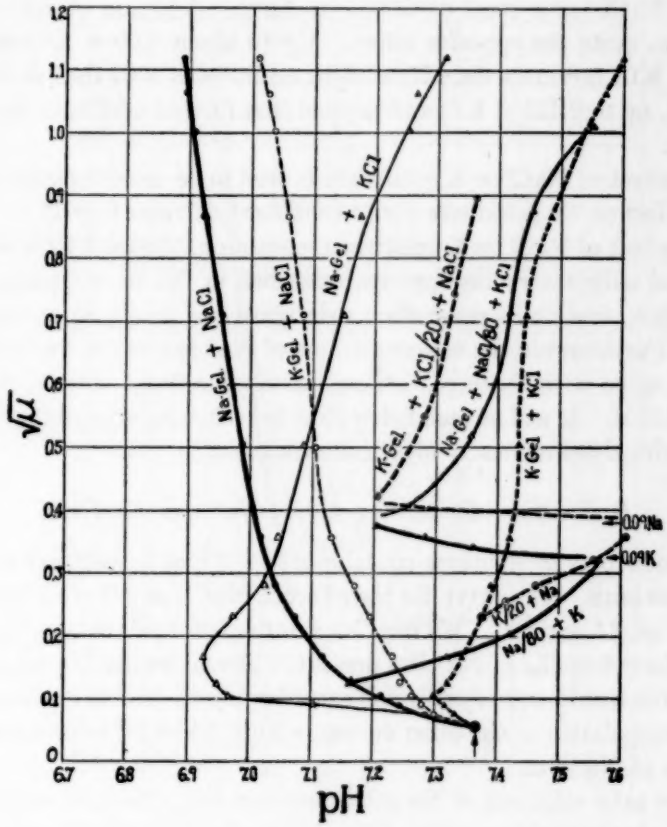

FIG. 1. Effect of NaCl and KCl on the pH of Na gelatinate and K gelatinate. We have plotted the square root of the total ionic strength, including that of the Na (or K) gelatinate (0.0032μ). The solid lines represent data with Na gelatinate; the broken lines with K gelatinate. The two lighter lines are for the effect of one type of chloride on the opposite type of gelatinate.

The Z-shaped curves are for solutions containing appreciable amounts of both cations, one being constant and the other varied.

gelatinate or to K gelatinate[7] (see Figs. 1 or 3A), although in the former case the initial decrease (up to 0.01 M K^+ ion) is much greater. Above 0.01 M K^+ ion concentration the two curves are seen to be parallel.

The Na gelatinate contains 0.0032 M Na^+ ion. In order to observe the effect of more Na^+ ion we then used Na gelatinate containing 0.0125 NaCl (or a total of 0.0157 M Na^+). Addition of KCl then produces quite the opposite effect. Up to about 0.09 M K^+ concentration KCl *increases* the pH; a slight addition of KCl then *decreases* the pH, up to 0.125 M K^+; and beyond that further additions *increase* the pH.

The effect of NaCl on K gelatinate is seen to be essentially the same as its effect on Na gelatinate (*i.e.*, a consistent decrease in pH).

The effect of NaCl on K gelatinate containing 0.050 M KCl is almost identical with the reverse process described in the second paragraph above (*i.e.*, first an increase, then a decrease and finally an increase in pH). Furthermore the concentrations of Na^+ ion at the two critical points agree with those of K^+ ion described above, namely, 0.09 M and 0.125 M. It will be seen below that the solutions containing Mg^{++} have critical points nearly identical with these.

B. Solutions Containing Mg^{++}, Na^+ and K^+ Ions.

We now pass to solutions containing Mg^{++} ions in addition to Na^+ or K^+ or both. The curve for the effect of $MgCl_2$ on gelatin is given in both Figs. 2A and 3A. We used Na gelatinate for this experiment and hence have 0.0032μ of Na^+ ion present. Should we use Mg gelatinate the curve would undoubtedly fall considerably to the left as shown by the extrapolation of the other curves in Figs. 2A or 2B to zero concentration of Na^+ ion.

If we take solutions of Na gelatinate and add a definite amount of $MgCl_2$ plus various amounts of NaCl, the effect up to 0.10 molar Na^+ ion is to *increase* the pH. At 0.10 molar there is a sharp break and a

[7] The K gelatinate was prepared like the Na gelatinate except that KOH was used instead of NaOH. The pH without salt was 7.203. Hence 0.164 was added to all these values plotted in Figs. 1 and 3 in order that the data might be compared with the Na gelatinate data.

slight further addition of NaCl causes a marked *decrease* in pH. At 0.15 molar Na^+ ion this effect ceases and further additions of NaCl have very slight effect on the pH. This is demonstrated by the lighter curves in Fig. 2A. In Fig. 2B are plotted the differences between these curves and the curve of NaCl alone (*i.e.*, the observed

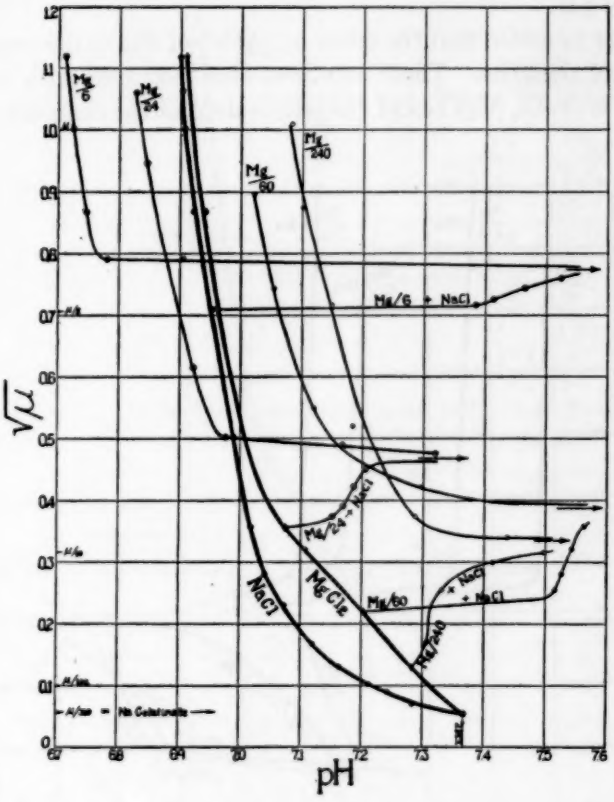

FIG. 2A. Effect of NaCl and $MgCl_2$ on Na gelatinate.

The square root of the total ionic strength is plotted against pH. The two heavy lines represent the effect of NaCl or $MgCl_2$ alone on Na gelatinate. In the latter case the presence of the 0.0032 M Na^+ ion has doubtless affected the curve. Extrapolation of the curves for the mixtures shows that in the absence of Na^+, $MgCl_2$ would cause a much more marked depression of the pH.

The lighter curves show the effect of addition of NaCl to Na gelatinate containing a fixed amount of $MgCl_2$.

pH with $MgCl_2$ and NaCl, minus the pH with NaCl alone at the same ionic strength). It is obvious that in the presence of $MgCl_2$ the addition of NaCl produces breaks in the curves at definite concentrations *which are independent of the amount of $MgCl_2$ present* (at least from 0.0042 to 0.17 M $MgCl_2$). Comparing the curves in the higher concentrations of NaCl, it is evident that in this range, addition of $MgCl_2$ *lowers* the pH.

It might be added that the effect on pH is not due to the sequence of addition of the salts. Three solutions were prepared with the same amounts of NaCl, $MgCl_2$ and Na gelatinate, but the components were

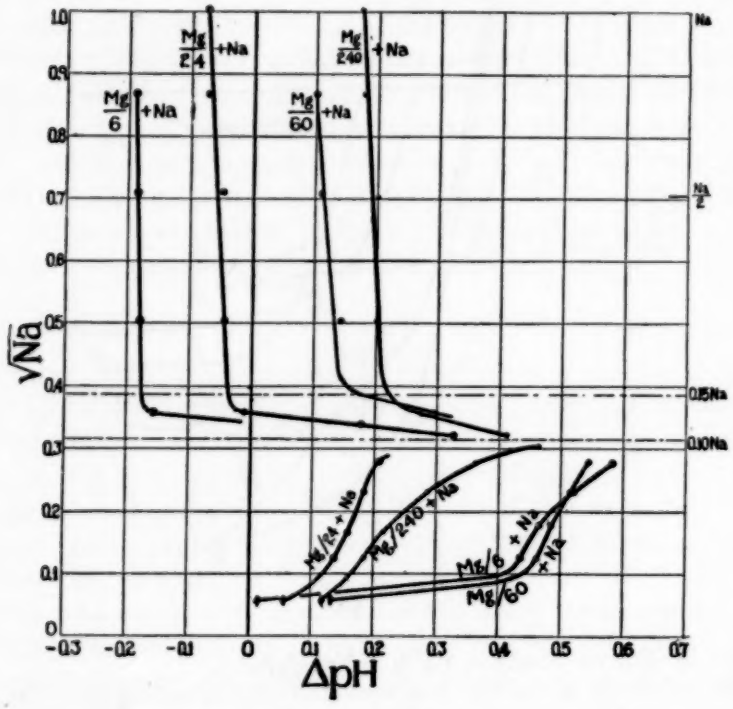

FIG. 2B. Effect of $Na^+ - Mg^{++}$ mixtures relative to that of NaCl alone.

The values for ΔpH represent the observed pH values minus the pH with the same ionic strength of NaCl. These are plotted against the square root of the Na^+ concentration. (The Mg^{++} is not included.) It is seen that the "breaks" are independent of the amount of $MgCl_2$.

mixed in different order in each case. All three had essentially the same pH.

The effect of KCl on gelatin containing $MgCl_2$ is almost identical up to 0.12 M K^+ ion with the effect of NaCl (compare Fig. 3A with

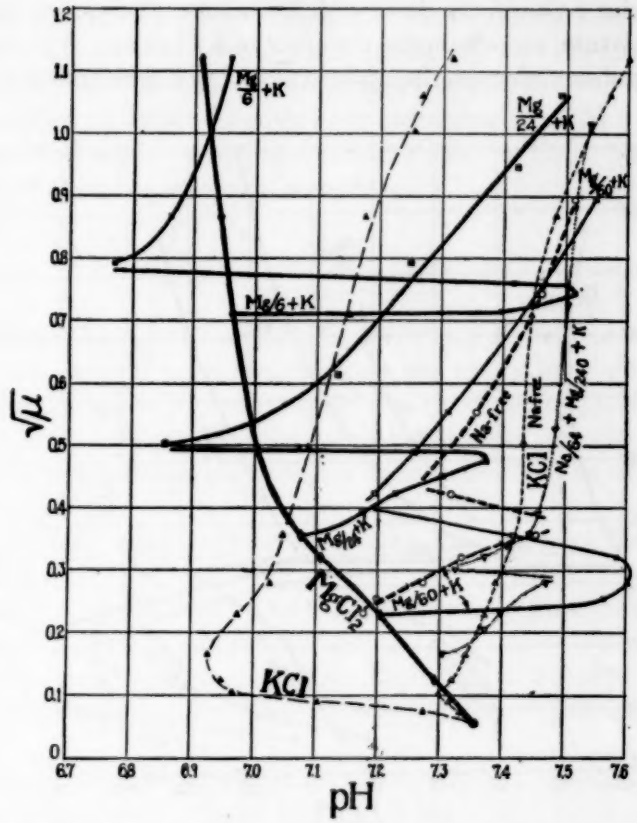

FIG. 3A. Effect of KCl and $MgCl_2$ on the pH of Na gelatinate and K gelatinate. Also the effect of a mixture of Mg, Na and K.

Curves are given for the effects of $MgCl_2$ alone on Na gelatinate, KCl alone on Na gelatinate and KCl alone on K gelatinate (indicated "Na-free").

The effects of KCl on Na gelatinate containing fixed amounts of $MgCl_2$ are represented by solid Z-shaped curves. Using K gelatinate gives the Z-shaped curve marked "Na-free" (dash line).

The effect of appreciable amounts of all three cations is given by the dotted line.

2A and Fig. 3B with 2B). Above the point of the second break (0.12 M K^+ ion) the curves in the case of KCl go to the right (higher pH) which is not surprising since KCl alone in higher concentrations causes an increase in pH.

In Fig. 3B we have plotted the difference between the curves with $MgCl_2$ plus KCl and the curve with KCl alone. This gives the effect of the mixture, and eliminates the effect of KCl alone. The dash line gives the observations on the addition of KCl to K gelatinate containing

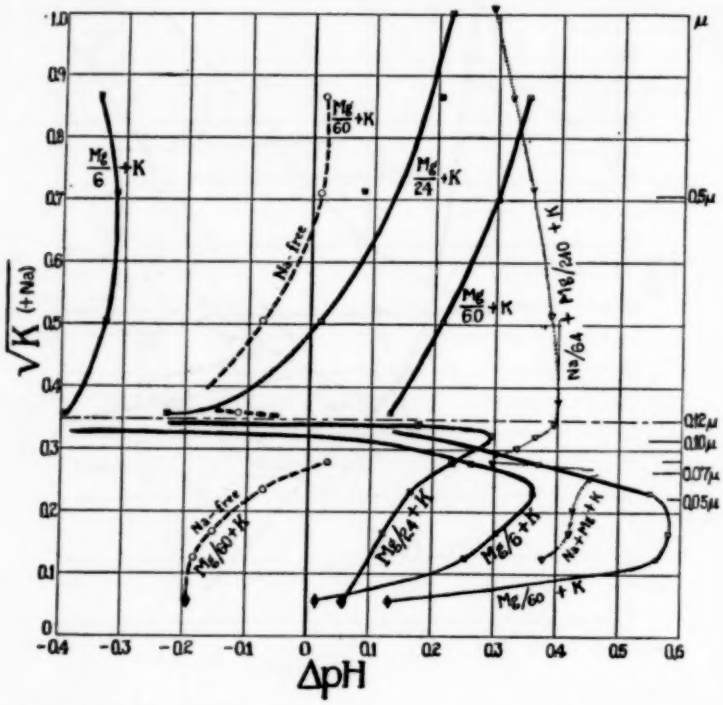

FIG. 3B. Effect of $K^+ - Mg^{++}$ mixtures or a $K^+ - Na^+ - Mg^{++}$ mixture, relative to that of KCl alone.

The values of ΔpH equal the observed pH values minus the pH with the same ionic strength of KCl (on Na gelatinate or K gelatinate as the case may be). These are plotted against the square root of K^+ *plus* Na^+ concentration. (The Mg^{++} is not included.) It is seen that the "breaks" are independent of the amount of $MgCl_2$. (*Cf.* Fig. 2B.)

$MgCl_2$. Since no Na^+ was present the effect of this ion does not enter in, but it will be seen that this curve is essentially like those in Fig. 2B (with Na gelatinate and NaCl).

The *heavy* lines in Fig. 3B (also 3A) represent data on Na gelatinate and hence contain 0.0032 M Na^+ ion. These are seen to be essentially like that with K gelatinate. The *dotted* line represents similar solutions with an added amount of NaCl, raising the Na^+ ion concentration to 0.0157 M. This curve is essentially like the others except that the critical points come at slightly lower concentration of $[Na^+ + K^+]$, namely at 0.07 M $[Na^+ + K^+]$ and at 0.08 M $[Na^+ + K^+]$. There is a third break at 0.12 M, but not a sharp one.

III.

DISCUSSION.

The above observations, while incomplete, may throw light on some of the physiological anomalies, such as the antagonistic effects of cations; the failure of Na^+ alone or K^+ alone to supplant a mixture of the two in physiological systems; and the unequal distribution of ions between the various physiological fluids, secretions and tissues in a given organism.

The essential qualitative generalizations to be made from the above observations are:

1. Mixtures of NaCl and KCl behave differently from either salt alone, in their effect on gelatin activity.

2. Mixtures of $MgCl_2$ and NaCl (or KCl) behave differently from these salts alone, the effect being slightly different if KCl is used in place of NaCl.

3. Mixtures containing all three cations, Mg^{++}, Na^+ and K^+ in appreciable quantities, behave similarly to Mg^{++} plus NaCl or KCl, but the curve is complicated by three breaks instead of two.

IV.

THEORY.

Before attempting to explain the above phenomena, we may remind the reader that previous observations[2,8] have shown a tendency of cations to affect the ionic activity of simple amines and ampholytes, in

[8] Simms, H. S., *J. Phys. Chem.*, 1929, xxxiii, in press.

a manner not in agreement with the Debye-Hückel theory and corresponding to the complex salts observed by Pfeiffer and collaborators.[9] These deviations with gelatin also agree with the combinations of ions with proteins found by Northrop and Kunitz[10] in a different manner.

It would seem that the various cations have an affinity for certain portions of the protein molecule but it is at first difficult to understand how two cations tending individually to produce one effect can produce the opposite effect in a mixture.

Let us consider the gelatin in this pH range to consist of two ionic species. The first, which we will call A, has its arginine group ionized, and predominates at lower pH values. The second, B, has its arginine group non-ionized, and predominates at higher pH.

Let us assume, for example, that in a given concentration, the tendency for Na^+ ion to combine with A is such as to produce negligible effect on its activity, while Na^+ reduces the activity of B to 0.9; then its effect on the arginine dissociation index (8.1) of gelatin would be represented by the logarithm of the ratio of γ_B/γ_A. The change in pH would in this range equal the change in pK; hence:

$$\Delta pH = \Delta\ pK' = \log \gamma_B/\gamma_A$$

or

$$\Delta pH = \log 0.9/1.0 = -0.05.$$

Let us assume furthermore that a given concentration of Mg^{++} ion reduces the activity of A to 0.5 and the activity of B to 0.3; then

$$\Delta pH = \log 0.3/0.5 = -0.22.$$

Thus Mg^{++} produces an effect in the same direction as Na^+.

Let us assume thirdly that in a mixture of the same amounts of Na^+ and Mg^{++} ions the species A is affected principally by Mg^{++} ion ($\gamma_A = 0.5$) and that B is affected principally by Na^+ ion ($\gamma_B = 0.9$). It is then obvious that the effect on the pH will be in the *opposite direction* from the effect with Na^+ or Mg^{++} alone, since

[9] Pfeiffer, P., and Wittha, F., *Ber. chem. Ges.*, 1915, xlviii, 1289. Pfeiffer, P., Würgler, J., and Wittha, F., *Ber. chem. Ges.*, 1915, xlviii, 1938; *Z. physik. Chem.*, 1924, cxxxiii, 22; cxxxiv, 180; cxxxv, 16; cxliii, 265.

[10] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1928, xi, 481.

$$\Delta pH = \log 0.9/0.5 = +0.25.$$

The first two of the above assumptions are legitimate and reasonable. The third assumption is made to agree with the observations. If it is correct it means that in a mixture of Na^+ and Mg^{++} these ions do not combine with the two ionic species of gelatin in the same ratio that they do when present alone, but that Na^+ combines more with one ionic species and Mg^{++} more with the other. (Although this might be accounted for with Na^+ and Mg^{++}at low concentrations on the basis of the charge, such an explanation would fail in the case of Na^+ and K^+.)

V.

EXPERIMENTAL.

2.50 per cent gelatin solutions were made up in 250 cc. amounts, and containing 16 cc. 0.100 M NaOH, or KOH. These were used as mother solutions in all measurements.

For each observation 5.00 cc. of mother solution was placed in a 10 cc. volumetric flask; the requisite amounts of the various salts were added, and the solution made up to 10.00 cc. (1.25 per cent gelatin). The salts were added volumetrically from stock solution in three concentrations (0.025μ, 0.25μ and 2.50μ).

The pH of each solution was measured at 25.0°C. in a water-jacketed hydrogen electrode of the bubbling type.[11]

The accuracy of the measurements needs mentioning It is difficult to get accurate readings on protein solutions in this range, but by frequent and careful plating of the electrodes, and by passing the hydrogen over red hot copper, it was possible to make readings reproducible in general to 0.0001 volt (0.001 pH). As an illustration, fresh mother solution with NaOH was made up six times, and each time the pH without salt was exactly 7.367. (Two solutions with KOH had the same pH of 7.302.)

The readings with salts were discarded if at least two consecutive readings out of three readings on each solution did not agree within 0.0002 volt (0.003 pH). Furthermore all data which from the nature of the curve seemed doubtful were repeated, and were discarded if they were not corroborated. New solutions were then observed with new electrodes.

The data are given in Tables I to XI.

[1] Simms, H. S., *J. Am. Chem. Soc.*, 1923, xlv, 2503

TABLE I.

Effect of NaCl on Na Gelatinate.

C_{NaCl}	pH	C_{NaCl}	pH	C_{NaCl}	pH
0	7.367	0.025	7.120	0.500	6.954
0.0025	7.284	.050	7.073	.750	6.921
.0050	7.243	.075	7.041	1.000	6.907
.0075	7.213	.125	7.018	1.125	6.903
.0125	7.164	.250	6.987	1.250	6.900

TABLE II.

Effect of KCl on Na Gelatinate.

C_{KCl}	pH	C_{KCl}	pH	C_{KCl}	pH
0	7.367	0.025	6.927	0.500	7.147
0.0025	7.283	.050	6.973	.750	7.176
.0050	7.103	.075	7.025	1.000	7.249
.0075	6.968	.125	7.046	1.125	7.267
.0125	6.949	.250	7.086	1.250	7.311

TABLE III.

Effect of KCl on K Gelatinate.

0.164 is added to the following pH values to give those plotted in Figs. 1 and 3A.

C_{KCl}	pH	C_{KCl}	pH	C_{KCl}	pH
0	7.203	0.025	7.178	0.500	7.281
0.0025	7.169	.050	7.209	.750	7.318
.0050	7.152	.075	7.225	1.000	7.374
.0075	7.135	.125	7.256	1.125	7.406
.0125	7.156	.250	7.269	1.250	7.436

TABLE IV.

Effect of NaCl on K Gelatinate.

0.164 is added to these pH values to give those plotted in Fig. 1.

C_{NaCl}	pH	C_{NaCl}	pH	C_{NaCl}	pH
0	7.203	0.025	7.068	0.500	6.921
0.0025	7.137	.050	7.032	.750	6.895
.0050	7.115	.075	7.005	1.000	6.873
.0075	7.099	.125	6.968	1.125	6.863
.0125	7.081	.250	6.941	1.250	6.846

TABLE V.

Effect of KCl on Na Gelatinate Containing 0.0125 M NaCl.

(pH without salt = 7.367)

μ for each solution equals $C_{KCl} + 0.0157$.

C_{KCl}	pH	C_{KCl}	pH
0	7.164	0.112	7.281
0.0125	7.318	.125	7.203
.025	7.419	.250	7.354
.050	7.499	.500	7.416
.075	7.549	.750	7.453
.087	7.563	1.000	7.548
.100	7.359		

TABLE VI.

Effect of NaCl on K gelatinate Containing 0.050 M KCl.

(pH without salt = 7.203)

0.164 is added to these pH values to give those plotted in Fig. 1.

μ for each solution equals $C_{NaCl} + 0.0532$.

C_{NaCl}	pH	C_{NaCl}	pH
0	7.213	0.125	7.042
0.0125	7.249	.250	7.117
.0250	7.298	.500	7.164
.0500	7.393	.750	7.198
.0750	7.446		

TABLE VII.

Effect of $MgCl_2$ on Na Gelatinate.

C_{MgCl_2}	pH	C_{MgCl_2}	pH	C_{MgCl_2}	pH
0	7.367	0.100	7.108	0.750	6.942
0.0125	7.293	.125	7.074	1.250	6.910
.050	7.203	.500	6.963		

TABLE VIII.

Effect of $MgCl_2$ Plus NaCl on Na Gelatinate Containing $MgCl_2$.
(pH without salt = 7.367)

C_{NaCl}	pH	Δ pH*	C_{NaCl}	pH	Δ pH*
A. 0.00416 M $MgCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.0157$)					
0	7.285	0.117	0.100	7.438	0.411
0.0125	7.305	.177	.125	7.287	.274
.0250	7.306	.216	.250	7.184	.201
.0500	7.331	.287	.500	7.149	.200
.0750	7.414	.373	.750	7.101	.177
.0875	7.497	.463	1.000	7.083	.174
B. 0.0167 M $MgCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.0532$)					
0	7.203	0.131	0.125	7.296	0.294
0.0125	7.516	.461	.250	7.120	.141
.025	7.526	.485	.500	7.054	.110
.050	7.543	.518	.750	7.021	.100
.075	7.563	.545			
C. 0.0416 M $MgCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.128$)					
0	7.074	0.057	0.112	7.167	0.178
0.0125	7.151	.138	.125	6.977	−.010
.025	7.169	.159	.250	6.922	−.045
.050	7.186	.184	.500	6.905	−.048
.075	7.205	.209	.750	6.845	−.072
.100	7.319	.328	1.000	6.827	−.076
D. 0.167 M $MgCl_2$ plus various amounts of NaCl ($\mu = C_{NaCl} + 0.503$)					
0	6.963	0.013	0.125	6.780	−0.156
0.0125	7.384	.436	.250	6.746	−.180
.025	7.413	.467	.500	6.724	−.185
.050	7.465	.521	.750	6.711	−.190
.075	7.523	.583	1.000		

* The values of ΔpH in this table equal the above pH value minus the pH of the same ionic strength of Na gelatinate plus NaCl (Fig. 1 or 2A).

TABLE IX.

Effect of KCl on Na Gelatinate Containing $MgCl_2$.
(pH without salt = 7.367)

A. 0.0167 M $MgCl_2$ plus various amounts of KCl ($\mu = C_{KCl} + 0.0532$)

C_{KCl}	pH	Δ pH*	C_{KCl}	pH	Δ pH*
0	7.203	0.131	0.125	7.193	0.130
0.0125	7.563	.560	.250	7.311	.212
.025	7.600	.580	.500	7.452	.300
.050	7.585	.550	.750	7.548	.347
.075	7.416	.370			

B. 0.0416 M $MgCl_2$ plus various amounts of KCl ($\mu = C_{KCl} + 0.128$)

0	7.074	0.057	0.125	6.856	−0.230
0.025	7.174	.120	.250	7.133	+.016
.050	7.227	.164	.500	7.250	.083
.075	7.301	.230	.750	7.421	.205
.100	7.371	.292	1.000	7.491	.220
.112	7.258	.176			

C. 0.167 M $MgCl_2$ plus various amounts of KCl ($\mu = C_{KCl} + 0.503$)

0	6.963	0.013	0.125	6.773	−0.394
0.0125	7.398	.252	.250	6.861	−.330
.025	7.450	.302	.500	6.927	−.316
.050	7.514	.360	.750	6.956	−.344
.075	7.416	.260			

* The values of ΔpH in this table equal the above pH values minus the pH of the same ionic strength of Na gelatinate plus KCl (Fig. 1).

TABLE X.

Effect of KCl on Na Gelatinate Containing 0.0125 M NaCl plus 0.00417 M $MgCl_2$.
(pH without salt = 7.367)
μ for each solution equals $C_{KCl} + 0.0282$.

C_{KCl}	pH	Δ pH*	C_{KCl}	pH	Δ pH*
0	7.305	0.378	0.100	7.441	0.394
0.0125	7.372	.420	.125	7.457	.400
.0250	7.398	.425	.250	7.484	.387
.0500	7.470	.452	.500	7.504	.354
.0625	7.323	.295	.750	7.514	.320
.0750	7.370	.334	1.000	7.538	.288
.0875	7.406	.363			

* The values of ΔpH in this table equal the above pH values minus the pH of the same ionic strength of Na gelatinate plus KCl (Fig. 1 or 3A).

TABLE XI.

Effect of KCl on K Gelatinate Containing 0.0167 M $MgCl_2$.
(pH without salt = 7.203)
0.164 is added to these pH values to give those plotted in Figs. 3A and 3B.
μ for each solution equals $C_{KCl} + 0.0532$.

C_{KCl}	pH	Δ pH*	C_{KCl}	pH	Δ pH*
0	7.014	−0.194	0.125	7.154	−0.113
0.0125	7.035	−.183	.250	7.193	−.078
.0250	7.073	−.153	.500	7.298	+.012
.0500	7.171	−.073	.750	7.350	+.020
.0750	7.289	+.030			

* The values of ΔpH in this table equal the above pH values minus the pH of the same ionic strength of K gelatinate plus KCl (Fig. 1 or 3A). It will be noted that these solutions are free from Na.

VI.

SUMMARY.

1.25 per cent gelatin solutions containing enough NaOH to bring them to pH 7.367 (or KOH to pH 7.203) were made up with various concentrations of NaCl, KCl and $MgCl_2$, alone and in mixtures, up to molar ionic strength. The effects of these salts on the pH were observed.

$MgCl_2$ and NaCl alone *lower* the pH of the Na gelatinate or the K gelatinate, in all amounts of these salts.

KCl first *lowers* the pH (up to 0.01 M K^+), then *raises* the pH.

Mixtures of NaCl and KCl (up to 0.09 M of the salt whose concentration is varied) *raise* the pH; then (up to 0.125 M Na^+ or K^+) *lower* the pH; and finally (above 0.125 M) behave like KCl alone.

Mixtures of $MgCl_2$ and NaCl *raise* the pH up to 0.10 M Na^+, and *lower* it up to 0.15 M Na^+ *regardless of the amount of* $MgCl_2$. Higher concentrations of NaCl have little effect, but the pH in this range of NaCl concentration is lowered with increase of $MgCl_2$.

Mixtures of $MgCl_2$ and KCl behave as above described (for $MgCl_2$ and NaCl) and the addition of NaCl *plus* KCl to gelatin containing $MgCl_2$ produces essentially the same effect as the addition of either alone, except that the first two breaks in this curve come at 0.07 M and 0.08 M $[Na^+ + K^+]$ and there is a third break at 0.12 M.

In this pH range the free groups of the dicarboxylic acids and of lysine are essentially all ionized and the prearginine and histidine groups are essentially all non-ionized. The arginine group is about 84 per cent ionized. Hence we are studying a solution with two ionic species in equilibrium, one with the arginine group ionized, and one with it non-ionized.

It is shown that the effect of each salt alone depends upon the effect of the cation on the activity of these two species due to combination. The anomalous effects of cation mixtures may be qualitatively accounted for if one or both of these species fail to combine with the cations in a mixture in proportion to the relative combination in solutions of each cation alone.

Special precautions were taken to ensure accuracy in the pH measurements. The mother solutions gave identical readings to 0.001 pH and the readings with salts were discarded when not reproducible to 0.003 pH. All doubtful data were discarded.

CHEMICAL AND PHYSICAL CHANGES IN GELATIN SOLUTIONS DURING HYDROLYSIS.

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The initial stages of the hydrolysis of gelatin solutions by either enzymes or acids are accompanied by a very marked decrease in the viscosity of the solution. In fact the viscosity may change 20 or 30 per cent before any chemical change can be noted unless very sensitive methods such as conductivity are used. It is not surprising, therefore, that the "liquefying" action of enzymes has been at times considered as purely physical and distinct from their hydrolytic action. As Waldschmidt-Leitz (1) has emphasized, however, all our knowledge of protein hydrolysis goes to show that the reaction is purely chemical and consists in the rupture of a peptide linking. Nevertheless, it would not be expected that such a reaction would cause any marked change in the viscosity, since viscosity, according to Einstein (2), is a function merely of the volume of the solute, and two small molecules should occupy nearly the same volume when separate as when they are combined. In many cases, such as the hydrolysis of sugar or of dipeptides or even of proteins having a low viscosity, this is true and the hydrolysis is accompanied by only slight changes in viscosity. In the case of gelatin, however, the change in viscosity is relatively enormous and it is evident that there is some secondary mechanism by means of which a very small chemical change gives rise to a very large physical one. The explanation of the viscosity of gelatin solutions assumed by Loeb (3) and extended by Kunitz (4) furnishes the required mechanism. According to this hypothesis a gelatin solution contains particles or micelles consisting probably of an insoluble material, enclosing and surrounded by a solution of soluble material. At about 10 per cent total concentra-

tion the concentration of the gelatin is the same inside and outside of the particles. Below 10 per cent the gelatin is more concentrated inside than outside the micells. When acid or alkali is added a Donnan equilibrium is set up between these micells and the surrounding solution and they swell, thereby increasing the viscosity of the solution. If salt is now added the Donnan effect is lessened, the micells shrink and the viscosity decreases. The same effect could be obtained by adding to the solution some material which could not penetrate the micells. When the total concentration is 10 per cent or higher the concentration of gelatin in the micells is the same as that of the outside solution and adding acid therefore has no effect. Let us assume now that the action of enzymes or of acid destroys the micells either by hydrolyzing the insoluble network or the internal solute. Both effects probably occur. In either event the result will be a marked decrease in the volume of the micells. A very slight chemical action will thereby cause a very large physical change. Under certain conditions there will be a further secondary effect. If the micells are swollen due to the presence of acid the material liberated from the destroyed micells will depress the swelling of the remaining intact ones just as would the addition of a salt. It would be expected therefore that the change in viscosity, or better, in the volume of solute, for a given amount of chemical change should be greatest when the viscosity is due to swelling of the micells, as in acid, and should be less at the isoelectric point, or when salt had been added, or in concentrated gelatin solutions.

In order to test this assumption gelatin was hydrolyzed by pepsin and the increase in formol titration and decrease in viscosity determined simultaneously.

The gelatin was prepared as previously described (5). The viscosity was determined by du Noüy's (6) torsion viscometer and the increase in the formol titration determined by the method described in a preceding paper (7). The experiments were carried out in a constant temperature bath at 37°C.

The results of the experiments are shown graphically in Figs. 1, 2 and 3, in which the decrease in viscosity has been plotted against the corresponding increase in formol titration. Fig. 1 gives the result of an experiment with 1 per cent gelatin at various pH values. The

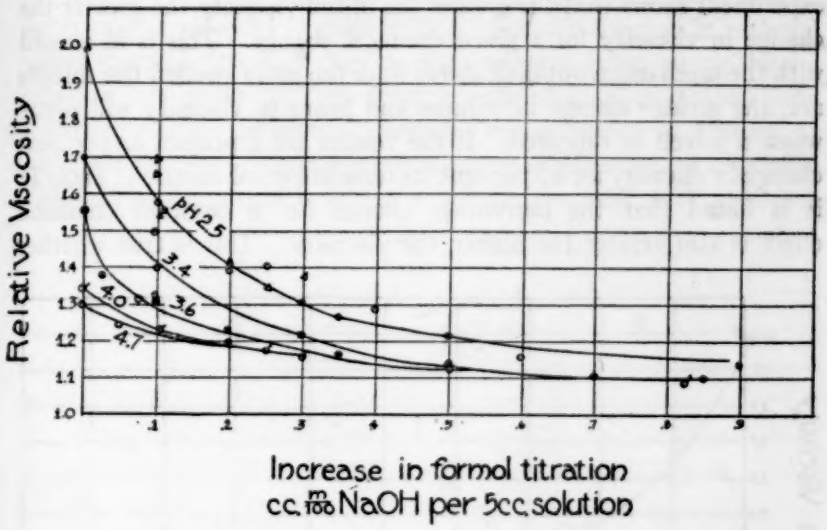

FIG. 1. Decrease in viscosity of 1 per cent gelatin solutions at various pH values, as a function of the increase in formol titration.

TABLE I.

Per Cent Change in Viscosity or Volume of Gelatin Solutions under Various Conditions Caused by 5 Per Cent Increase in Formol Titration.

Concentration of gelatin..	1 per cent							8.5 per cent		
pH.....	4.7	4.0	3.7	3.4	3.0 or 2.5	3.0	3.0	4.7	4.0	3.5 or 3.0
Salt concentration...	0	0	0	0	0	0.1M	0.4M	0	0	
Original viscosity (H_2O = 1.0).....	1.30	1.35	1.60	1.70	2.0	1.45	1.35	13.0	14.0	15.0
Final viscosity.....	1.19	1.19	1.23	1.29	1.4	1.26	1.21	6.0	6.8	7.0
Per cent decrease in viscosity actual....	8	12	23	24	30	14	10	54	51	53
(corrected for H_2O).....	37	46	62	59	60	42	40	59	55	60
Original volume gelatin cc. per 100 cc. solution.....	5.8	6.5	10.0	11.3	14.5	8.0	6.5	44.7	45.6	46.5
Final volume gelatin.	3.9	3.9	4.6	5.6	7.3	5.1	4.3	33.4	35.4	35.8
Per cent decrease in volume.....	33	40	54	50	50	36	34	26	23	23

experiment shows that the greater the initial viscosity the greater the change in viscosity for a given chemical change. This is in accord with the mechanism outlined above since the more swollen the micells are, the greater change in volume and hence in viscosity will occur when a micell is ruptured. If the results are expressed as per cent change in viscosity for a 5 per cent increase in formol titration (Table I) it is found that the percentage change for a constant chemical effect is also greater the higher the viscosity. This is true whether

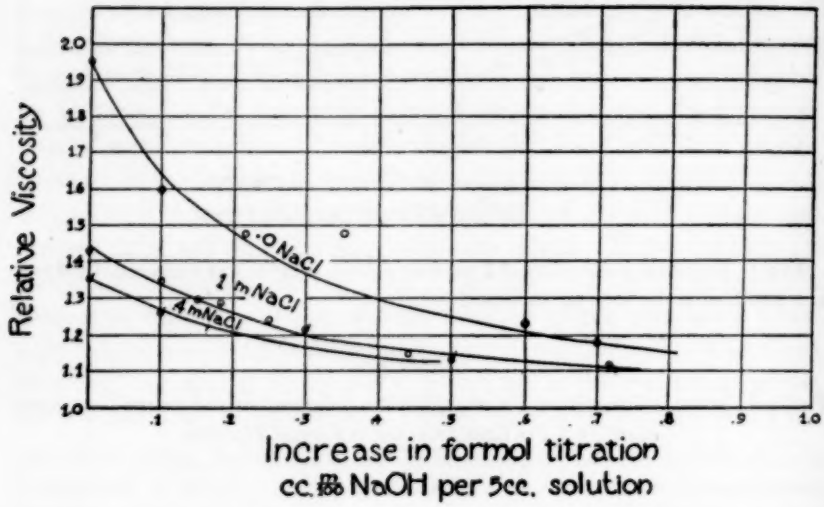

FIG. 2. Decrease in viscosity of 1 per cent gelatin at pH 3.0 with increasing salt concentration, as a function of the increase in formol titration.

the actual viscosity or the viscosity corrected for that of water is used. If, instead of the viscosity, the change in volume according to Kunitz's (8) equation corresponding to this viscosity change is used, the result is the same except that the percentage differences are not so marked. The fact that the percentage change in viscosity is greater the greater the initial viscosity is due to the secondary effect mentioned above. If the action consisted simply in the rupture of the micells the percentage loss of volume of the micells due to the rupture of the same number should be the same, and should be independent of the size of the micells. This is not the case, since when

the micells are swollen and the viscosity therefore high, as at pH 3.0, the change in viscosity is greater for a 5 per cent increase in carboxyl groups (formol titration) than at the isoelectric point where the micells are less swollen. This effect is probably due, as stated above, to the influence of the material liberated by the rupture of a micell on the other micells. When the micelles are swollen due to a Donnan equilibrium the presence of more soluble material will result in

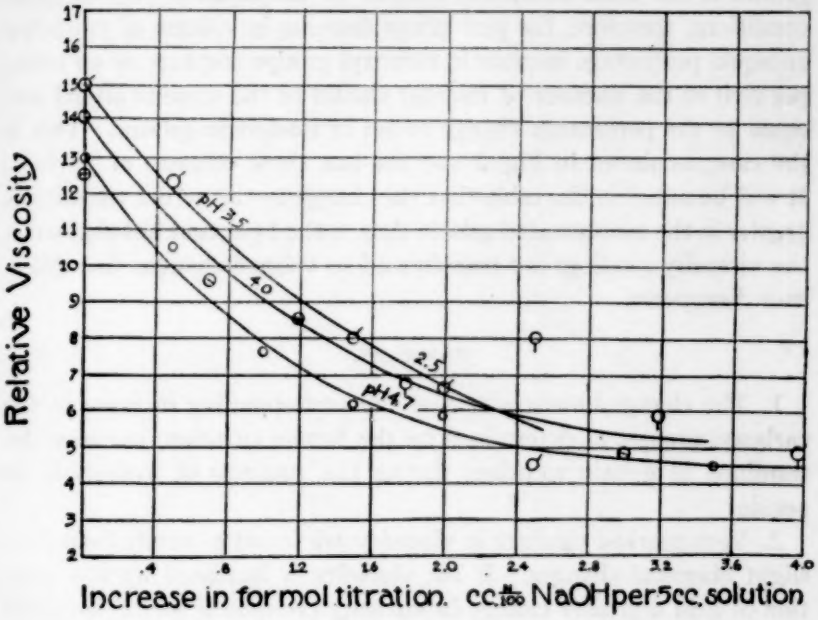

FIG. 3. Decrease in viscosity of 8 per cent gelatin at various pH values, as a function of the increase in formol titration.

a decrease in the osmotic pressure due to this equilibrium and the micells will therefore shrink. At the isoelectric point, where there is no Donnan equilibrium and the micells are less swollen, the material liberated from the ruptured micells will have little or no effect on the intact ones. It could also be assumed that, when the micells are highly swollen, less chemical hydrolysis is necessary to rupture them. Probably both mechanisms are at work.

If the swelling of the micells at pH 3.0 is repressed by the addition of salt evidently the change in viscosity for the same percentage change in the number of carboxyl groups should be the same as at the isoelectric point, and this is the result as shown in Fig. 2 and Table I.

At a concentration of gelatin of about 8 per cent the effect of adding acid on the viscosity practically disappears and this has been explained by Kunitz (4) on the assumption that the concentration of gelatin is the same inside and outside of the micells. Under these conditions, therefore, the percentage decrease in volume of solute for an equal percentage increase in carboxyl groups (rupture of an equal per cent of the number of micells) should be the same at all pH and equal to the percentage change found in isoelectric gelatin. This is the case, as shown in Fig. 3 and the last three columns of Table I. It will be noted in the table that the change in the actual viscosity is greater in the concentrated gelatin than in the 1 per cent but that when the viscosity readings are transformed to volume changes this difference disappears.

SUMMARY.

1. The change in viscosity and the corresponding increase in the carboxyl groups, as determined by the formol titration, has been determined in gelatin solutions during the progress of hydrolysis by pepsin.

2. Very marked changes in viscosity are found to result from very slight chemical changes. If the viscosity is increased by the addition of acid a greater change in viscosity (volume of solute) is caused by the same percentage change in the number of carboxyl groups. The percentage change in the volume of solute, caused by the same percentage increase in the number of carboxyl groups, is independent of the concentration of gelatin.

3. These results are in agreement with the idea that the high viscosity of gelatin solutions is due to the presence of swollen micells, since a slight chemical hydrolysis may be sufficient to rupture a micella and so cause a very large change in viscosity.

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THE SWELLING OF GELATIN AND THE VOLUME OF SURROUNDING SOLUTION.

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The theory of the swelling of gelatin in acid as developed by Procter and Wilson and by Loeb (1) is based on the Donnan equilibrium and assumes that the swelling is due to the osmotic pressure caused by the unequal distribution of the ions of the acid inside and outside of the gelatin. At equilibrium this osmotic pressure for strong monovalent acids is given by the expression

(1) $$2Y + Z - 2X$$

X = concentration acid in supernatant liquid.
Y = " free acid in gelatin.
Z = " non-diffusible ion = concentration Cl^- equivalent to ionized protein.

It follows that with pure gelatin at equilibrium in acid the swelling depends only on the *concentration* of acid outside the gelatin and is independent of the *volume* of the acid. The equilibrium concentration of acid and hence the swelling will depend, however, on the quantity as well as the concentration of the acid originally taken. The quantitative statement of this effect is complicated and has been discussed by Hitchcock (2). Qualitatively, however, the result may be simply predicted. When the swelling of gelatin in HCl is plotted against the pH of the acid there is found a maximum at about pH 3.0. If increasing volume of acid solution having a pH of 3.0 or higher is added to a series of constant amounts of gelatin, therefore, the pH of the solution at equilibrium will approach the initial pH of the acid as more acid is added. The swelling will therefore increase with increasing amount of acid solution and finally reach a maximum constant value corresponding to the pH of the pure acid. This will be true as soon as the amount

of acid which combined with the gelatin is negligible compared to the total amount of acid added. If acid of greater strength than pH 3.0 is used the pH will decrease again as the amount of acid solution added is increased and will again become constant when the amount of solution added is so large that there is no change in pH on addition of the gelatin. The swelling will now increase with the volume of solution, reach a maximum (at pH 3.0) and then decrease to a constant value. Since it is necessary for equilibrium that there be some excess solution, *i.e.*, that sufficient solution is used so that some remains after the gelatin is swollen, it would be possible with very strong acid to bring the gelatin to a pH less than 3.0 before there is any excess solution. In this case, after enough solution has been added to furnish an excess of liquid the swelling will decrease as the amount of acid solution added increases. If the swelling obtained in the different series is plotted against the volume of the original acid solution used, therefore, the swelling will increase, decrease or pass through a maximum with increasing volume of solution, depending on the original concentration of the acid solution. Such results have been recorded in the literature (3). If, however, the swelling values from these experiments are plotted against the pH of the solution (either of the gelatin or supernatant solution) at equilibrium, then the swelling should be independent of the volume of solution. According to Küntzel (3), however, this is not the case. Küntzel found that even when the swelling was plotted against the pH of the solution at equilibrium, the swelling increased with increasing volume of the solution. This result is contrary to that expected from the theory for the case of pure acid and gelatin, but is the expected result if the *gelatin contained neutral salts*. The swelling is depressed by neutral salts and hence the less the volume of acid added the greater the depressing effect of the salt. The swelling even at constant pH will therefore increase with the volume of acid added until the salt is so dilute as to have no effect. This is the result obtained by Küntzel (3). Küntzel used gelatin without previous purification and hence salts were present. In order to be sure of this explanation, however, experiments similar to Küntzel's were done with ordinary gelatin and also with isoelectric gelatin. The results with isoelectric gelatin are given in Fig. 1 in which the swelling at equilibrium has been plotted against the volume

of acids of different concentration added. As predicted, the swelling with dilute acids increases with the quantity of acid. The swelling in M/50 acid passes through a maximum while stronger acid gives decreasing swelling with increasing volume. When these curves are plotted against pH at equilibrium (either of the gelatin or outside

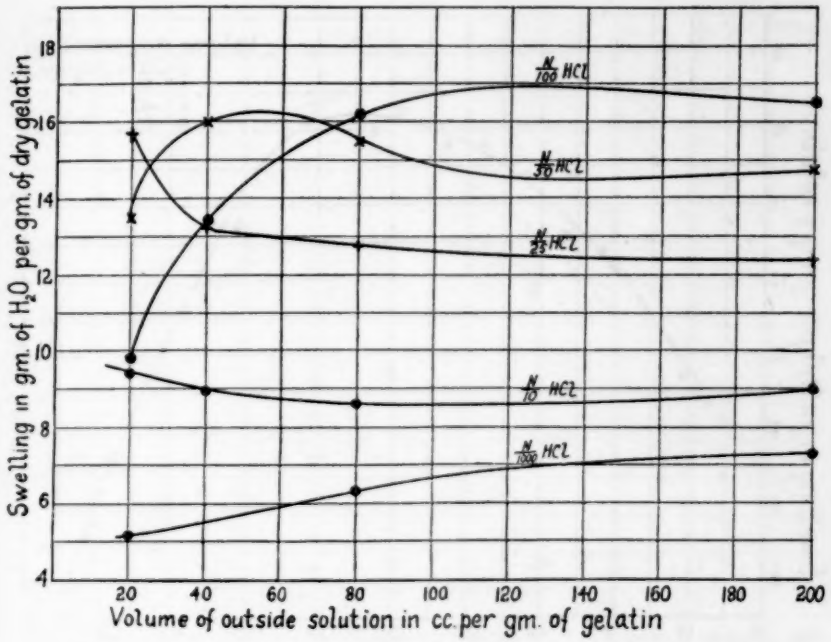

FIG. 1. Swelling of gelatin as determined by the volume of acid of various concentrations.

solution) they coincide as shown in Fig. 2, giving the usual pH swelling curve for gelatin in HCl. When the experiment is performed with unpurified gelatin the results shown in Fig. 3 are obtained which agree with those of Küntzel. As stated above the increase in swelling with increased volume in this case is due to the decreased salt concentration. It may be mentioned that the absolute amount of swelling cannot be compared in experiments done with different samples of gelatin since this property varies markedly in different preparations.

Experimental Procedure.

Cooper's powdered gelatin was used. The isoelectric gelatin was prepared as described previously (4). The experiments were carried out in a water bath at 5°C. 200 cc. of acid was placed in a series of 500 cc. Erlenmeyer flasks and brought to the temperature of the bath. The flasks were equipped with mechanical stirrers which rotated at approximately the same speed. The gelatin was then

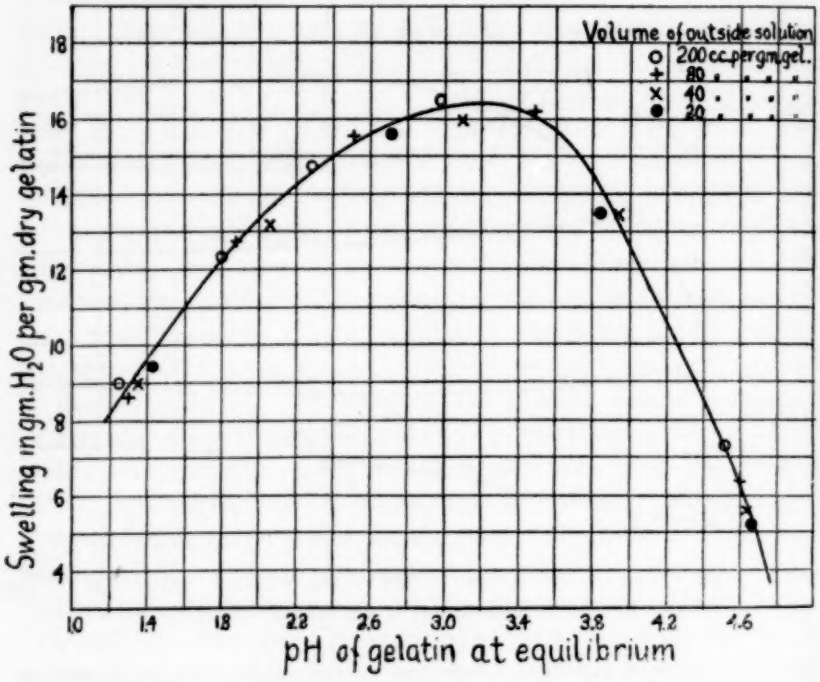

FIG. 2. Swelling of purified gelatin with increasing volume of acid solution as a function of the pH of the gelatin.

added and the suspensions stirred for 5 hours. Preliminary experiments showed that equilibrium was reached in about 2 hours. The gelatin was then filtered off with suction through coarse filter paper and the concentration of the swollen gelatin determined by drying a weighed sample to constant weight at 100°C. Dry weight determinations were also made on the supernatant solution. No appreciable amount of gelatin was found in the supernatant. The pH of the melted gelatin and of the outside solution were determined electrometrically at 30°C.

It may be pointed out that a number of sources of error must be guarded against in swelling experiments of this type. If the temperature is much above 5° there will be some solution of the gelatin and this will increase with the acidity and volume of the solution. The final volume depends to a slight extent on the time required for the swelling to take place, since a true equilibrium is never reached but the mass of gelatin continues slowly to swell indefinitely. If precautions

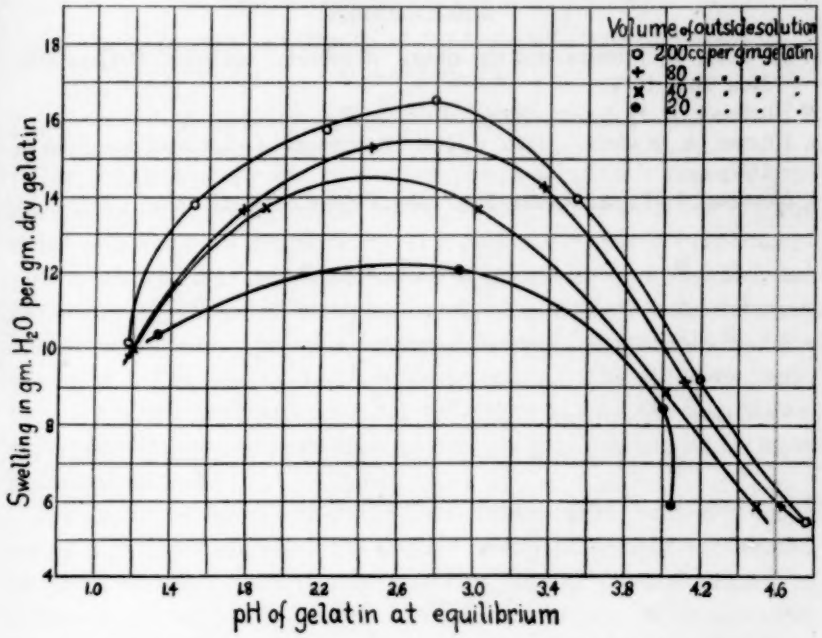

FIG. 3. Swelling of gelatin containing electrolytes with increasing volume of acid solution as a function of the pH of the gelatin.

for rapid stirring are not taken, therefore, the gelatin in a large volume of acid will swell more slowly than that in a small volume and the figure obtained for the equilibrium value will be slightly different.

SUMMARY.

The swelling of isoelectric gelatin added to various volumes of acid of different concentration at 5°C. has been determined. The swelling is determined only by the concentration of the supernatant solution at equilibrium and is independent of the volume of acid.

Similar experiments with *unpurified* gelatin show that in this case, owing to the presence of neutral salts the swelling is a function of the volume as well as the concentration of acid.

Both results are predicted by the Procter-Wilson-Loeb theory of the swelling of gelatin.

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A METHOD FOR THE DETERMINATION OF DIFFUSION CONSTANTS AND THE CALCULATION OF THE RADIUS AND WEIGHT OF THE HEMOGLOBIN MOLECULE.

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The diffusion coefficient of a substance in solution is a useful constant since from it may be calculated, by Einstein's equation (1), the radius and weight of the particle. It is particularly useful in the case of substances which cannot be obtained in pure solution and which are frequently of interest in biological work. In such cases the diffusion coefficient is the only practical means of determining the molecular size or weight. The classical method for determining the diffusion coefficient is so beset with experimental difficulties and, in the case of slow moving substances, requires such a long time that it is useless for most biological material.

It occurred to the writers that if the diffusion could be made to occur across a thin porous plate the process would be greatly accelerated owing to the high concentration gradient, and at the same time the elaborate precautions to prevent convection currents, etc. inherent to the classical method would be eliminated (2). Search of the literature revealed that several attempts (3–6) had already been made in this direction, but none were practical for biological material owing to the unsuitable nature of the membranes used. It was found, after a number of trials, that rapid and reproducible results could be obtained with thin membranes made either of alundum or glass powder.

Theory of the Method.—The diffusion coefficient is defined as the quantity of material that will diffuse across a plane of unit dimensions in unit time under unit concentration gradient, or

$$D = \frac{dQ}{A\ dt \frac{dc}{dx}} \tag{1}$$

where D is the diffusion coefficient, dQ is the quantity which passes across the plane of area A in time dt under a concentration gradient of dc/dx. There are a number of solutions of this differential equation depending on the conditions of the experiment (*cf.* Mellor (7)). The present case is in many respects the simplest. Suppose a solution of concentration C_1 is separated from a more dilute solution of concentration C_2 by a porous membrane through the pores of which the solute can diffuse. Both solutions are stirred so that diffusion occurs only across the membrane. The solute will diffuse from C_1 to C_2 and if the volume of these solutions is relatively large and the experiment carried on for a short time the concentrations will remain practically constant.* Let the effective area of the pores be A and the effective

* The equation for diffusion, under the above conditions, for the general case in which the concentration cannot be considered constant during the duration of the experiment, may be derived as follows:

Let

S = total solute at beginning of experiment
v_1 = volume concentrated solution
v_2 = volume dilute solution
Q = quantity solute in dilute solution
$= c_2 v_2$
$S - Q = c_1 v_1$

From equation (1)

$$\frac{dQ}{dt} = \frac{DA}{h}(c_1 - c_2)$$

substituting for c_1 and c_2

$$= \frac{DA}{h}\left(\frac{S - Q}{v_1} - \frac{Q}{v_2}\right)$$

or

$$= \frac{DA}{h\, v_1\, v_2}(v_2 S - (v_2 + v_1) Q)$$

Integrating, substituting $K = \frac{h}{A}$

$$D = \frac{2.3\, K\, v_1\, v_2}{(v_1 + v_2)\, t} \log \frac{v_2 S - (v_2 + v_1) Q_0}{v_2 S - (v_2 + v_1) Q}$$

where $Q = Q_0$ when $t = 0$.

length (the distance through which the solute diffuses) be h. The concentration gradient will then be constant and equal to $\frac{C_1 - C_2}{h}$ and the quantity Q diffusing in time t will be simply

$$Q = D A t \frac{C_1 - C_2}{h} \tag{2}$$

or

$$D = \frac{h Q}{A t (C_1 - C_2)} \tag{3}$$

If the experiment is so arranged that the dilute solution is originally pure solvent, C_2 is zero and the equation is still further simplified to

$$D = \frac{h}{A t} \frac{Q}{C_1} \tag{4}$$

Dimensions of D.—Since concentration may be expressed as quantity per unit of volume, the units used to measure the quantity cancel out provided the same unit is used to express the concentration as is used to measure the quantity diffusing. D therefore reduces to area over time, or if time is expressed in days and length and volume in centimeters, to cm.^{2} per day. This may be seen from the following equation

$$D = \frac{h \text{ cm.}}{A \text{ cm.}^2} \frac{Q \text{ units}}{t \text{ day} \dfrac{Q_1 \text{ units}}{\text{cm.}^3}} = \frac{h Q \text{ cm.}^2}{A Q_1 t \text{ day}} \tag{5}$$

in which Q_1 is the number of units per cc. of the concentrated solution. If the amount contained in 1 cc. of the concentrated solution is taken as the unit of quantity, *i.e.* if $Q_1 = 1$, and the amount diffused is expressed in this unit (*i.e.* as the number of cc. of the concentrated solution containing the quantity diffused), the equation may be still further simplified and written

$$D = \frac{h Q_{cc.} \text{ cm.}^2}{A t \text{ day}} = \frac{K Q_{cc.} \text{ cm.}^2}{t \text{ day}} \tag{6}$$

where $Q_{cc.}$ is the number of cc. of the concentrated solution that contains the amount of substance diffused. For instance, if it were found in an

experiment with HCl that 10 cc. of 0.10 acid had diffused and the concentrated solution were 0.10 M acid then Q_{∞}. would be 10. If the concentrated solution had been 1.0 M, Q_{∞}. would be 1.0, etc. Any comparative unit of quantity may therefore be used to express the quantity diffused provided the concentration is expressed in the same units and this is an advantage in dealing with biological material.

It is evident that in order to obtain the diffusion coefficient in absolute units it is necessary to know the dimensions of the membrane through which the diffusion occurs. In the case of porous membranes this value cannot be measured directly since the effective radius and the arrangement of the pores is not known. For any one membrane, however, the effective thickness and area may be assumed constant and therefore h/A is constant and may be called K, the membrane constant. In order to obtain this value it is necessary to standardize the apparatus against some solution the diffusion constant of which is known, just as is the case with a conductivity cell. K, the cell constant, may then be found from the equation

$$K = \frac{D\,t}{Q_{cc.}} \tag{7}$$

When this constant has been determined for a particular membrane it may then be used to determine the diffusion coefficient for unknown substances, provided of course that the effective pore area is the same for the standard and for the unknown.

Construction of the Apparatus.—It follows from the preceding considerations that the following conditions must be fulfilled by the apparatus.

1. The concentration of the two solutions on opposite sides of the membrane must be kept constant, by stirring or otherwise, so that diffusion occurs only across the membrane.

2. The quantity allowed to diffuse must be small enough so that the difference in concentration between the two sides may be considered constant during the experiment.

3. The membrane must be thick enough so that the liquid in the pores is not disturbed by the stirring of the solutions. On the other hand, the thinner it is the more rapidly the experiment can be completed.

4. The pores must be small enough to prevent convection currents in the liquid held in them and large enough to allow free diffusion of the particles (or molecules) of the solute.

5. The membrane must be level in order to prevent flow from one solution to the other.

It was found after a number of trials that porous glass or alundum discs gave satisfactory results.

The Membranes.—Filter discs made by pressing together a uniform powder of Jena glass are obtainable from the Jenaer Glaswerk, Schott and Gen., Jena, Germany—American agent J. E. Bieber, 1123 Broadway, New York. Discs of the porosity now called No. 4, but formerly designated as <7, were used. According to the manufacturers the pore diameter is 5 to 10 microns or thousandths of a millimeter. The radius of the hemoglobin molecule is about 2.7×10^{-3} microns. The discs are cut down to approximately the desired diameter with an ordinary hack saw and rounded off exactly on a carborundum wheel. Then the pores of the membrane are filled with melted rosin by gentle suction applied through the stop-cock of the glass vessel to which the disc is to be attached. This is to prevent the clogging of the pores with the carborundum powder with which the disc is next ground down to about 0.5 mm. thickness. At the beginning the grinding is done on a carborundum stone, and finished on a flat piece of glass. The rosin is removed with a suitable solvent such as chloroform.

The glass discs are usually sold already fused on to vessels of various shapes, one of them very like the one used in these experiments. It is better, however, to start with the loose discs, since as a result of the fusion the effective area of the disc is lessened.

The membranes are attached to the glass vessel with de Khotinsky cement applied to the bottom of the vessel. Then the edges of the discs which may stick out slightly here and there are carefully ground down even with the vessel and covered with cement. Leaks are tested for by blowing through the stop-cock while the membrane is under water.

Alundum filter discs, porosity RA 225, are made by the Norton Company, Worcester, Massachusetts. The disc is ground down to the desired diameter on a wheel and to the desired thickness by being rubbed against another disc. At the finish a fresh flat disc should be used. After the powder produced in the grinding is washed out the disc is attached.

Some batches of alundum discs contain air spaces large enough to cause small holes when the discs are ground thin. Such discs cannot be used.

Assembly of the Apparatus.—The apparatus is assembled as shown in Fig. 1. The cell contains about 20 cc. and the outside solution is 10 to 50 cc. depending on the rate of diffusion, the time of the experiment, and the analytical method used. The cell is filled by gentle suction through the membrane and rinsed with water. It is then clamped in position and leveled by means of a small level or by placing a dish of mercury just below it. The membrane is made parallel to the mercury by adjusting the universal clamp. The mercury is then removed and replaced with sufficient pure solvent to cover the membrane. The time is noted and the diffusion allowed to continue until the desired amount has passed through

the membrane. The outside solution is then pipetted off and analyzed and replaced by a new portion. This process is continued until the quantity passing through the membrane is constant for equal time intervals, *i.e.*, the system is in the steady state. This constant figure is then used. If more than a few per cent of the solute diffuses out it is necessary to correct the concentration of the inside solution by this amount. The amount diffusing during any one determination should be not more than 2 or 3 per cent of the total in the concentrated solution, and under these conditions a sufficiently accurate correction is obtained by considering the concentration as the average value for the duration of the experiment. For instance, if it is found that 4 per cent of the solute has dialyzed out during the experiment, then the concentration of the inside solution has dropped from 100 to 96 during the experiment. For purposes of calculation it is considered to have remained constant at 98 per cent.

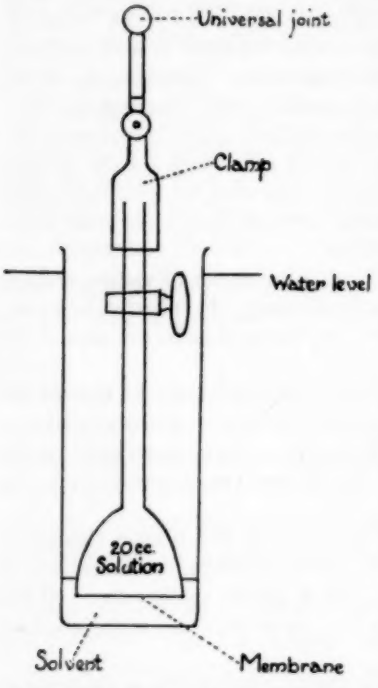

FIG. 1. Apparatus for determination of diffusion coefficients.

The arrangement shown in which the heavier solution is above obviates the necessity of stirring since, as the solute diffuses through, a solution is formed near the membrane which is heavier than the pure solvent and so sinks to the bottom. This process could be clearly observed in the experiments with hemoglobin. As the solution in contact with the upper side of the membrane becomes more dilute it is replaced by the heavier concentrated solution above it. There are also slight convection currents due to temperature effects and vibrations from the stirrer of the water bath which aid this process. The fact that even with HCl the quantity diffusing remains constant for constant time intervals shows that the two solutions must remain nearly homogeneous, *i.e.*, that diffusion occurs only across the membrane as is assumed in the calculation. Any *flow* across the membrane would result in variable results in different experiments and would cause an increase in the relative amount of solution passing the membrane with more concentrated solutions.

Evidently the change in concentration due to the diffusion would be less if the volumes were larger. However, a larger volume of the cell increases the error caused by temperature fluctuations and a large volume of outside solution renders

the analysis more difficult. The apparatus is placed in a water bath at 5°C. since the coefficient of expansion of water is near the minimum at this temperature and also since biological material is more stable at this temperature. The temperature regulation was obtained by placing an ordinary electrically regulated glass water bath in a refrigerating room which had a temperature of about 3° to 4°C.

Calibration of the Apparatus.—It was stated above that since the actual dimensions of the membrane are not known it is necessary to standardize the apparatus by means of a solution whose diffusion coefficient is known. HCl, lactose, and several salts were used for this purpose and it was found that the same cell constant was obtained for any particular membrane with all substances. The results with HCl will be given as an example.

The results of the experiment are shown in Table I. It was found that the constant for the cell was 0.148. The physical significance of this figure, as may be seen from equation (6), is evidently the ratio of effective thickness to pore area of this membrane.

TABLE I.

Diffusion of HCl through Alundum Membrane 1, 5°C.

Solution	Time t	Equivalent cc. 0.1 N HCl diffused	Average
	days	$Q_{cc.}$	
0.1 N HCl	0.0208	0.262	
	.0208	.258	0.261
	.0208	.262	

$$K = \frac{Dt}{Q_{cc.}} = \frac{1.85 \times 0.0208}{0.261} = 0.148$$

D = diffusion coefficient 0.1 N HCl at 5°, extrapolated from Ohölm's data = 1.85 cm.^{2} per day.

It is now possible to determine the diffusion coefficient of another substance by means of this membrane. Carbon monoxide hemoglobin was chosen for this purpose since the molecular weight of hemoglobin is known from osmotic pressure measurements and the weight calculated from the diffusion experiment may therefore be checked against this value.

Preparation of Hemoglobin.—Whipped beef blood is saturated with CO, cooled, and washed three times with cold isotonic salt solution which is likewise saturated with CO. An equal volume of water is added to the corpuscles. The laked corpuscles are shaken with a quarter their volume of toluene (Heidelberger (10)) and the mixture allowed to stand in the cold overnight. The toluene and cell debris are removed by centrifuging. By dilution with 1/20 M phosphate or borax buffers the hemoglobin is brought to the desired concentration and hydrogen ion concentration. It is filtered through fine filter paper and saturated with CO.

During the experiments the hemoglobin is allowed to diffuse into buffer solution saturated with CO.

The carbon monoxide hemoglobin which has diffused through the membrane is estimated colorimetrically.

Carbon monoxide hemoglobin was used since it is the most stable form of hemoglobin.

TABLE II.

Determination of Diffusion Coefficient of Hemoglobin, 5°C.

Membrane	Hemoglobin solution	Time	Hemoglobin diffused as cc. concentrated solution	K for membrane	D hemoglobin cm.²/day
		days	$Q_{cc.}$		
Alundum 1	1 per cent pH 6.8	0.75	0.202	0.150	0.0405
		.92	.264		.0430
		2.06	.558		.0407
	2.5 per cent pH 6.8				.0430
					.0412
					.0428
Alundum 2	2.5 per cent COHb pH 6.8			.168	.0401
	2.5 per cent COHb M/20 borax pH 9.3				.0397
Glass 1	2.5 per cent COHb pH 6.8			.100	.0434
Glass 2	2.5 per cent COHb pH 6.8 M/20 phosphate			.0833	.0456
Average					0.0420 ± 0.0005

Diffusion Coefficient of Hemoglobin.—The results of several diffusion experiments are given in Table II. The table gives the amount of hemoglobin found to have diffused through various membranes after various time intervals and from different hemoglobin solutions. This quantity has been expressed as the number of cc. of the concentrated solution (used for the experiment), which contained the same amount

of hemoglobin. The diffusion coefficient of hemoglobin may therefore be calculated from these figures by means of equation (8).

$$D = \frac{K\,Q_{cc.}}{t} \tag{8}$$

The values obtained in this way are given in the last column of the table. The average value is 0.0420 ± 0.0005 cm.² per day.

Calculation of the Radius and Molecular Weight of the Molecule from the Diffusion Coefficient.—According to Einstein the diffusion coefficient is related to the radius of the molecule by the following equation

$$D = \frac{R\,T}{N} \cdot \frac{1}{6\,\pi\,r\,\eta} \tag{9}$$

$R = 8.3 \times 10^{7}$ erg · deg.$^{-1}$ · mole^{-1}

$N = 6.06 \times 10^{23}$ mole^{-1}

$T = 278$ deg. — for 5°C.

r = radius of particle including water of hydration, in cm.

η = viscosity of water at 5°C.

$= 0.01519$ gm. cm.$^{-1}$ sec.$^{-1}$

$= 0.01519$ erg · sec. cm.$^{-3}$

$$D = \frac{1.33 \times 10^{-13}}{r} \frac{\text{erg} \cdot \text{deg.} \cdot \text{mole} \cdot \text{cm.}^{3}}{\text{erg} \cdot \text{deg.} \cdot \text{mole} \cdot \text{cm.} \cdot \text{sec.}}$$

$$= \frac{1.33 \times 10^{-13}}{r} \frac{\text{cm.}^{2}}{\text{sec.}}$$

or for H_2O at 5°C.

$$= \frac{1.148 \times 10^{-8}}{r} \frac{\text{cm.}^{2}}{\text{day}} \tag{10}$$

therefore

$$r = \frac{1.148 \times 10^{-8}}{D} \tag{11}$$

$$= 2.73 \times 10^{-7} \text{ cm.}$$

The molecular weight of the particle may be calculated from the radius by the following equation provided the specific gravity is known.

$$m = \frac{4}{3} \pi r^3 g N \tag{12}$$

g = specific gravity = gm. cm.$^{-3}$

$= 1.33$

$= \frac{4}{3} \times 3.14 \times 6.06 \times 10^{23} g r^3$

$= 25.4 \times 10^{23} \cdot g \cdot r^3$

$= 25.4 \times 10^{23} \times 1.33 \times 20.2 \times 10^{-21}$

$= 68{,}500 \pm 1{,}000$

The specific gravity is assumed to be the same as that of solid hemoglobin and the calculation gives a molecular weight of 68,500 ± 1,000 which agrees within the experimental error with that of 67,000 found by Adair (8) by osmotic pressure measurements, and of 68,000 found by Svedberg (11) from experiments on the rate of sedimentation.

Einstein's equation assumes that the solute molecules are large compared to the solvent molecules and therefore could not be used for substances of small molecular weight. It was found by Euler (9), however, that for such molecules the diffusion coefficient is inversely proportional to the square root of the molecular weight, *i.e.*

$$\frac{D_1}{D_2} = \sqrt{\frac{m_2}{m_1}} \tag{13}$$

In such cases the molecular weight could be obtained by simple proportion from the diffusion constant of some known substance of about the same molecular weight.

Sources of Error in the Method and Assumptions Underlying the Theory.—The effect of temperature, leveling, and change of concentration during the experiment have already been mentioned. It is conceivable that the substance might stick to the membrane and so fill up the pores, or that some of the pores would not allow the unknown solution to pass while the solution used as a standard could pass. For this reason it is advisable to use different types of membrane since it is

unlikely that the effect would be the same in membranes made of different material. Another method of testing for this error is to determine the effect of filtering the solution through the membrane. Any appreciable adsorption would be shown by a change in concentration on filtering.

Einstein's equation for calculating the radius and molecular weight from the diffusion coefficient is based on the following assumptions. The diffusing particles (molecules) are few and large compared to those of the solvent. They are spherical and are impelled by a force equal to the osmotic pressure as given by Van't Hoff's law against a resistance as given by Stokes' law. The work of Perrin and others has shown that Einstein's equation is valid for a number of colloidal solutions. The specific gravity of the particle, however, is more uncertain since it might very well consist partially of water and so be lighter than the dry solid in bulk.

In the case of ionized substances another complication is introduced since the diffusion rate of one ion is influenced by the accompanying ion. Most biological substances, however, are weak electrolytes so that the quantity present in the form of ions is usually small. It is conceivable in the case of such things as enzymes that several different size molecules might be determined simultaneously by the method of analysis used. In this case no constant diffusion coefficient would be obtained since the smaller molecules would diffuse first. If the proportion or the relative number and sizes of the different molecules were known this effect could be corrected for, at least in theory. Practically, however, decreasing coefficients found on succeeding diffusions would indicate a mixture of substances but could hardly be analyzed further in a quantitative way. The problem is evidently the same as the separation of gases by diffusion.

Finally, it may be said that the molecular weight obtained in this way is that defined by the properties of the solution and used by the physicist and physical chemist.

SUMMARY.

A method is described for determining the diffusion coefficient of solutes by determining the rate of passage of the solute through a thin porous membrane between two solutions of different concentration.

The method has been used to determine the diffusion coefficient of carbon monoxide hemoglobin. This was found to be 0.0420 ± 0.0005 cm.^{2} per day at 5°C.

The molecular weight of carbon monoxide hemoglobin calculated by means of Einstein's equation from this quantity is 68,600 ± 1,000.

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OSMOSIS OF LIQUIDS. II.

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INTRODUCTION.

We take an osmotic system

$$n_1 \times L_1 \mid n_1' \times L_1' \qquad (1)$$

in which on the left side of the membrane are n_1 quantities of a liquid L_1 and on the right side n_1' quantities of L_1'. We now assume, in the same way as in the preceding communication,[1] that the system contains three substances, *viz.* X, Y and W (W = water); then we may represent L_1 and L_1' by the points 1 and 1′ (Fig. 3, Paper I). The position of their complex e, as we have seen previously, is determined by:

$$e1 : e1' = n_1' : n_1 \qquad (2)$$

When the membrane transmits only W, during the osmosis L_1 will proceed along the line 1.4 (Fig. 3, Paper I) and L_1' along the line 1′.4′. At last the osmotic equilibrium forms:

$$n_4 \times L_4 \mid n_4' \times L_4' \qquad (3)$$

Then the water diffusion has stopped; the two liquids are situated on the same isotonic W-curve ab and consequently they have the same O.W.A.

The phenomena are different, however, when the membrane transmits two or three substances. Here we shall discuss only the membrane that transmits the three substances.

[1] Schreinemakers, F. A. H., *J. Gen. Physiol.*, 1928, xi, 701.

I.

The Theoretical Osmosis Path.

We represent the liquids of system (1) by the points 1 and 1′ in Fig. 1; in order to save place, the sides and angle points of the triangle WXY have been omitted. The complex of both the liquids is represented by point e; its position has been determined by (2).

As soon as the osmosis has begun and the substances X, Y and W diffuse through the membrane in some direction or other, the liquids change their compositions. After a time t_2 the left side liquid has arrived *e.g.* in point 2 and the right side liquid in point 2′ (Fig. 1). As the quantities of the liquids have also changed now, we represent them by n_2 and n_2'. Consequently system (1) has now passed into:

$$n_2 \times L_2 \mid n_2' \times L_2' \tag{4}$$

Of course the total quantity of the two liquids remains constant during the osmosis; we have, therefore: $n_2 + n_2' = n_1 + n_1'$. The total composition of the system also remains constant; consequently the line 2.2′ also runs through point e. Instead of (2) we now have however:

$$e2 : e2' = n_2' : n_2 \tag{5}$$

After a time t_3 the left side liquid has arrived in a point 3 and the right side liquid in a point 3′; we then have a system:

$$n_3 \times L_3 \mid n_3' \times L_3' \tag{6}$$

For this we obtain:

$$n_3 + n_3' = n_2 + n_2' = n_1 + n_1'$$

The line 3.3′ also runs through point e; we now have:

$$e3 : e3' = n_3' : n_3 \tag{7}$$

Of course the same obtains for all systems that have formed during the osmosis. At the end of the osmosis the two liquids must be in osmotic equilibrium with one another. If only one substance passes through the membrane, then, as we have seen in Paper I, the two final liquids are different. If, however, the membrane is permeable with

respect to all substances, then the same final liquid must form on both sides of the membrane;[2] it is clear that this liquid is represented by point e. At the end of the osmosis, therefore, we get the system:

$$L_e \mid L_e \qquad (8)$$

During the osmosis the left side liquid, therefore, proceeds along a curve $1.2.3.\ldots e$, the right side liquid along a curve $1'.2'.3'.\ldots e$; the arrows indicate the direction in which the liquids proceed along these curves. We call these curves "the osmosis path" of the left and right side liquids; together they form the "osmosis path" of the system. In Figs. 2 and 4 osmosis paths have been drawn, which have

FIG. 1

been determined experimentally and will be more fully discussed later on. We shall call liquids, present at the same moment on both sides of the membrane (*e.g.* 1 and 1′ or 2 and 2′ etc.), "conjugated" liquids; the points representing those liquids are called conjugated points; the lines, uniting two conjugated points (*e.g.* 1.1′ or 2.2′ etc.) are called conjugation lines. Therefore, the conjugation lines of an osmosis path all run through a point e, which represents the composition of the complex and consequently of the final liquid too. Reciprocally every

[2] We here assume that no unmixing into 2 or more liquids will occur in the system.

straight line, going through point e, is a conjugation line; the points of intersection represent two liquids, to be found at a certain moment on both sides of the membrane.

The osmosis path of system (1) starts in Fig. 1 from the points 1 and 1′; its form, however, depends on several other factors, some of which we are going to discuss.

(*a*) The form of the path depends on $n_1:n_1'$ *viz.* on the ratio of the quantities of the liquids, which are brought on both sides of the membrane. For if we alter this ratio, it will be seen from (2) that the position of final point e on line 1.1′ also changes; we imagine this *e.g.* in q (or r). Of course the new osmosis path which must now run through q (or r) has another form than the path running through point e. Consequently an infinite number of paths start from the points 1 and 1′ determined by the ratio $n_1:n_1'$; we are able to prove that these paths touch one another in the points 1 and 1′.

(*b*) The form of the path depends on the direction in which and the velocity with which the substances diffuse through the membrane at any moment; consequently this form also depends on the nature of the membrane used and will, therefore, differ with different membranes. Later on we shall make this clear with some examples and discuss it in connection with other phenomena.

II.

The Experimental Osmosis Path.

If we leave an osmotic system

$$n_1 \times L_1 \mid n_1' \times L_1' \tag{9}$$

in which the two liquids and their quantities have been absolutely determined, alone, then it will change in a definite way. At a moment t_2 we then have a system

$$n_2 \times L_2 \mid n_2' \times L_2' \tag{10}$$

in which again the two liquids and their quantities have been completely determined. Consequently the system proceeds along a definite path (1.2.3.*e* and 1′.2′.3′.*e* of Fig. 1); we call this the "theoretical" path. If, however, in order to find out the composition of the

liquids of (10) we take away a little of these liquids, then we have instead of (10) the new system:

$$(n_1 - q) \times L_1 \mid (n_2 - q') \times L_2' \qquad (10a)$$

q and q' represent the quantities, taken away. Although (10) and (10a) consist of the same liquids, they still have another final liquid as a rule. Of course that of (10) is situated in point e; that of (10a) is situated in a point h on the conjugation line 2.2′ (Fig. 1).

In accordance with (5) for e we obtain:

$$e2 : e2' = n_2' : n_1$$

For point h we obtain, however:

$$h_2 : h_{2'} = (n_2' - q') : (n_1 - q)$$

Consequently e and h are different points, unless accidentally:

$$q : q' = n_1 : n_2'$$

System (10) which we no longer have, would, therefore, proceed along the paths 2.3.e and 2′.3′.e; system (10a) which we do have proceeds, however, along the paths 2.h and 2′.h which have not been drawn; consequently both systems proceed along different paths, which touch one another, however, in the points 2 and 2′. (*Cf.* paragraph (*a*).) Of course the conjugation lines of system (10) all run through point e, those of (10a) through point h.

If we take in (10) *e.g.*, $n_2 = 240$ and $n_2' = 260$, then we have the system:

$$240 \times L_1 \mid 260 \times L_2'$$

If we take away 5 gm. on both sides, then we get:

$$235 \times L_1 \mid 255 \times L_2'$$

For the points e and h we obtain, therefore:

$$e2 : e2' = 260 : 240 = 1.083 : 1$$
$$h2 : h2' = 255 : 235 = 1.085 : 1$$

In Fig. 1 point h must be situated very closely to e; consequently the paths of both systems will practically coincide. As we have to

take away a little of the liquids now and then for the experimental determination of a path, we consequently do not define the theoretical path, but a succession of parts of theoretical paths; they pass into one another continually in the points which have been determined experimentally. We call this path the "experimental." The less we change the ratio of the two liquids in taking away little quantities of them, the less the experimental will differ from the theoretical path.

III.

The Path of the Membrane.

As the substances X, Y and W diffuse, the membrane must also contain these three substances; consequently we may say that the membrane contains a liquid, the composition of which changes continually during the osmosis. If in Fig. 1 we represent this liquid by a point, then, during the osmosis, this proceeds along a curve ($1''.2''.e''$ Fig. 1); therefore ,we call this the "path of the membrane."

Consequently we have to distinguish three paths, *viz.* the path of the left side liquid ($1.2.e$), the path of the right side liquid ($1'.2'.e$) and the path of the membrane ($1''.2''.e''$). Of course the final liquid e'' of the membrane generally has another composition than the final liquid e. Therefore, the theoretical and experimental paths of a system depend besides upon the quantity and the composition of the liquid absorbed by the membrane. If, however, we have a small and thin membrane and much liquid, this influence may be neglected; if, however, we have a large or thick membrane, this influence may be very strong. We shall refer to this later on.

IV.

Some Examples.

Now we shall discuss some paths which we have determined in the systems:

$$\text{Water} \mid L_1' \qquad (11)$$

The right side liquid consists of:

$$X = NaCl \qquad Y = Na_2CO_3 \qquad W = H_2O$$

On the left side of the membrane is pure water, which, however, takes in X and Y at once and passes into a ternary liquid. The membrane of these systems consisted of a pig's bladder, which had been degreased with ether. The results of the determinations[3] are found in the Tables I–IV. In the first column of I–III we find the number of the determination under t, the time, *viz.* the number of hours after

TABLE I.

	t	X	Y	X	Y
1	0	0	0	6.991	9.021
2	7	1.263	1.423	5.802	7.732
3	19	2.242	2.388	4.992	6.950
4	31	2.739	3.109	4.536	6.452
5	51	3.316	3.533	4.195	6.123
6	91	3.646	4.216	3.877	5.665
7	118	3.682	4.410	3.803	5.437

TABLE II.

	t	X	Y	X	Y
1	0	0	0	6.729	9.057
2	17	1.246	0.387	5.663	8.268
3	29	2.756	1.163	4.557	7.430
4	41	3.589	1.930	3.876	6.856
		——q		——q'	
5	69	3.823	2.763	†3.762	6.400
6	101	†4.179	3.547	3.792	5.988
7	143	4.138	4.319	3.843	5.688
8	311	3.934	5.260	3.901	5.378

the beginning of the osmosis. We find in the third and fourth columns under X the amount of NaCl and under Y the amount of Na_2CO_3 of the left side liquid; in the fifth and sixth columns we find the same for the right side liquid. The concentrations have been given in per cent of weight. As the W amount of the liquids follows at once from the X and Y amounts, this has not been given.

[3] They have been done in collaboration with G. M. A. Kayser and L. J. van der Wolk.

System I (Table I).—If with the aid of Table I we draw the osmosis path of this system, we get a path such as has been represented schematically by $W2e$ and $1'.2'.e$ in Fig. 2. The left side path We is situated below, the right side path $1'e$ above the conjugation line $W1'$. We see from Table I and Fig. 2 that the X and Y amount of

TABLE III.

	t	X	Y	X	Y
1	0	0	0	9.222	8.976
2	4	0.377	0.087	8.781	8.811
3	12	1.223	0.334	8.024	8.489
4	24	2.366	0.695	7.149	8.056
5	36	3.319	1.054	6.494	7.685
6	54	4.391	1.640	5.852	7.211
7	78	5.212	2.417	5.431	6.702
		q		q'	
8	108	†5.666	3.258	5.262	6.206
9	145	5.651	4.051	†5.260	5.903
10	193	5.551	4.660	5.278	5.650
11	240	5.478	5.024	5.308	5.534

TABLE IV.

	X	Y	*Membrane*	*Type*
1 (I)	6.991	9.021	H^a	I
2	7.363	9.194	H^b	I
3	9.238	9.165	H^c	I
4 (II)	6.729	9.057	VI^g	II
5	12.006	7.019	VI^i	II
6 (III)	9.222	8.976	VI^h	III
7	9.227	8.984	VI^d	II or III
8	4.681	10.107	VI^e	II or III

the left side liquid continually increases during the osmosis; that of the right side liquid decreases. Calculating the W amount, we find that the W amount of the left side liquid continually decreases during the osmosis; that of the right side liquid increases.

We may represent the progress of the osmosis still in another way. For this purpose we plot the time t of the osmosis on a horizontal axis

(Fig. 3) with the aid of Table I; on the vertical axis we may plot either the X, the Y or the W amount of the two liquids. If we plot the X amount, we get an $X.t$ diagram such as has been drawn schematically in Fig. 3. The fully drawn curve O.2.3.e obtains for the left side liquid, the dotted curve $1'.2'.3'.e$ for the right side liquid. The final point e of the two paths is situated at infinite distance; consequently both paths approach one another asymptotically. With the aid of Table I the reader can easily draw the $Y.t$ and $W.t$ diagram of this system himself. We can also summarize the previous considerations in a scheme.

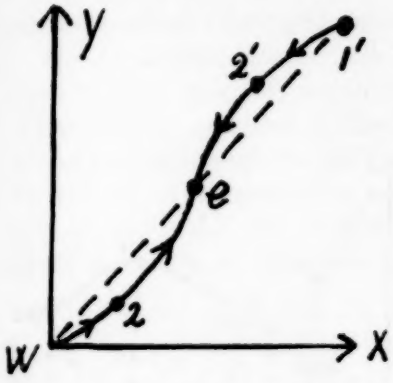

FIG. 2

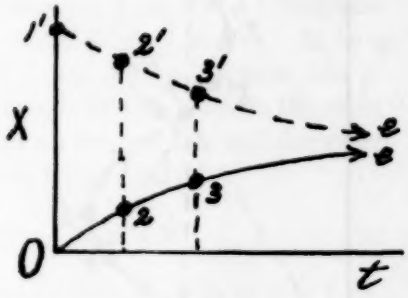

FIG. 3

Scheme I.

X	Y	W	
$\uparrow < \downarrow$	$\uparrow < \downarrow$	$\downarrow > \uparrow$	(12)

This means (*cf.* Paper I) that the left side liquid has a smaller X and Y amount, but a larger W amount than the right side liquid. An arrow pointing upwards indicates that the concentration increases; an arrow pointing downwards indicates that the concentration decreases. This shows therefore, that during the osmosis the X and Y amounts of the left side liquid increases, that of the right side liquid decreases; the W amount of the left side liquid decreases, that of the right side liquid increases. Consequently during the osmosis the liquids change (Paper I) their X, Y and W amounts normally.

System II (Table II).—If we draw the osmosis path of this system with the aid of Table II, we get the curves *W.q.*5.6.*e* and 1′.*q*′.5′.6′.*e* of Fig. 4; the *X.t* diagram has been represented in Fig. 5. For the sake of clearness both figures have been strongly schematised.

It appears from Table II that during the osmosis the X amount (consequently NaCl) of the left side liquid at first increases till the determination marked † (*viz.* 4.179 per cent NaCl) and decreases afterwards. Consequently in the vicinity of $n^\circ = 6$ the left side liquid gets a maximum X amount; we shall plainly say that this maximum coincides with point 6. Consequently the left path *Wq*56*e* must have

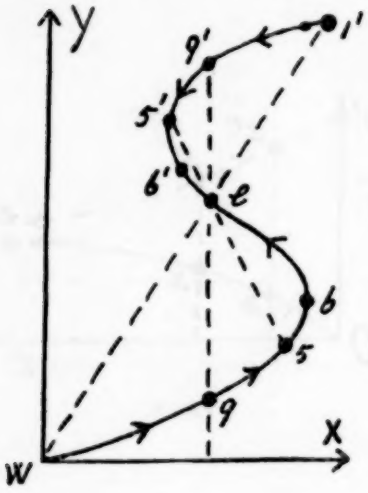

FIG. 4

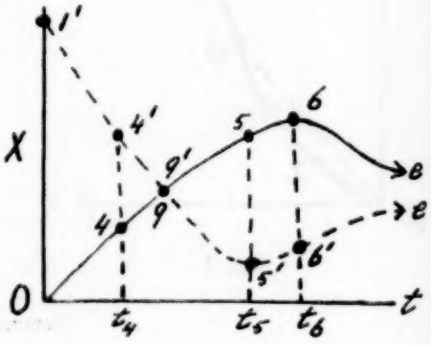

FIG. 5

a vertical tangent in point 6 of Fig. 4; in point 6 of Fig 5 this tangent is horizontal. The X amount of the right side liquid at first decreases till the determination marked † (*viz.* 3.762 per cent NaCl) and afterwards increases. Therefore, in the vicinity of $n^\circ = 5'$ the right side liquid gets a minimum X amount; again we assume that it coincides with point 5′. The right path 1′ *q*′ 5′ 6′ *e* must, therefore, have a vertical tangent in point 5′ of Fig. 4 and a horizontal tangent in point 5′ of Fig. 5. It also appears from Table II that between the determinations 4 and 5 the left side and the right side liquids have had the same X amount. This has been indicated in the table by the hori-

zontal lines beside which the letters q and q' have been placed. Consequently in Fig. 4 the conjugation line qeq' is vertical; of course in Fig. 5 q and q' are situated in the point of intersection of the two curves.

It appears from Table II or Fig. 4 that the changes of the Y and W amounts (*viz.* Na_2CO_3 and H_2O) during the total osmosis may be represented by

$$\begin{matrix} Y & & W \\ \uparrow < \downarrow & & \downarrow > \uparrow \end{matrix} \qquad (13)$$

just as in system I the liquids, change their Y and W amounts normally.

The X amount, however, changes in an absolutely different way from that in system I; we see this at once from the $X.t$ diagrams (Figs. 3 and 5) and from the osmosis paths (Figs. 2 and 4). As long as the system remains in the part Wq (and $1'q'$) of its path, the X amount on the left side of the membrane is smaller than on the right side; as during the osmosis it increases on the left side and decreases on the right side, we consequently have the symbol:

$$Wq\ (1'.q') \qquad \uparrow < \downarrow \qquad \text{(Figs. 4, 5)} \qquad (14)$$

So in this part of the path the X amount changes as in system I *viz.* normally-normally.

If, however, the left side liquid comes in q and the other consequently in q', both get the same X amount. As this increases on the left side of the membrane and decreases on the right side, we represent it by:

$$q\ (q') \qquad * \uparrow \!=\!=\!= \downarrow * \qquad \text{(Figs. 4, 5)} \qquad (15)$$

As we may consider the change on both sides of the membrane as abnormal, an asterisk has been placed with both arrows.

So here we have the same X amount on both sides of the membrane; during further osmosis this changes again, becoming equal once more in point e at the end of the osmosis.

When the system is in part $q5$ (and $q'5'$) of its path, then the X amount on the left side is greater than that on the right side. As,

however, it is still increasing on the left side and decreasing on the right side, we have the symbol:

$$q5\ (q'5') \qquad \bullet\ \uparrow > \downarrow\ \bullet \qquad \text{(Figs. 4, 5)} \tag{16}$$

Consequently the X amount changes abnormally here on both sides.

When the system is in point 5 (and 5′) then the X amount on the right side of the membrane (*viz.* in point 5′) is a minimum; therefore, this X amount does not change in an infinitely small time dt; on the

SCHEME II.

		X	Y	W
Wq	(Wq')	$\uparrow < \downarrow$	$\uparrow < \downarrow$	$\downarrow > \uparrow$
q	(q')	$\bullet\uparrow = \downarrow\bullet$	"	"
$q5$	$(q'5')$	$\bullet\uparrow > \downarrow\bullet$	"	"
5	$(5')$	$\bullet\uparrow > \vert\bullet$	"	"
5.6	$(5'.6')$	$\bullet\uparrow > \uparrow$	"	"
6	$(6')$	$\bullet\vert > \uparrow$	"	"
$6.e$	$(6'.e)$	$\downarrow > \uparrow$	"	"

left side of the membrane it increases, however. We represent this by:

$$5\ (5') \qquad \bullet\ \uparrow > \vert\ \bullet \qquad \text{(Figs. 4, 5)} \tag{17}$$

in which the dash indicates that the right side X amount does not change in a time dt.

When the system is in part 5.6 (and 5′.6′) of its path, the X amount increases on both sides. Consequently we have the symbol:

$$5.6\ (5'.6') \qquad \bullet\ \uparrow > \uparrow \qquad \text{(Figs. 4, 5)} \tag{18}$$

Consequently, the X amount changes abnormally here on the left side and normally on the right side.

If we also consider the system in point 6 (and 6′) and in part $6.e$ (and $6'.e$) of its path and combine all symbols, we get scheme II. From this we learn among other things the following: At the beginning of the osmosis the X amount of the left side liquid is smaller, afterwards (in q and q') equal, next larger and at the end of the osmosis (point e) again equal to that of the right side liquid. The X amount of the

left side liquid increases, beginning at point W as far as 6, and decreases afterwards as far as e. The X amount of the right side liquid decreases, starting from point 1′ as far as 5′, and increases afterwards as far as e. The X amount of the left side liquid changes abnormally from q to 6, for it increases, although it is already greater than that of the other liquid. The X amount of the right side liquid changes abnormally from q' to 5′, for it decreases although it is already smaller than that of the other liquid. On part 5.6 (and 5′.6′) of the path the X amount increases on both sides of the membrane.

System III (Table III).—In many respects this system resembles the preceding one, the following things namely appear from Table III. Between the determinations 7 and 8 the liquids get the same X amount; the X amount of the left side liquid becomes a maximum in the vicinity of the liquid marked † (*viz.* 5.666 per cent NaCl); the X amount of the right side liquid becomes a minimum in the vicinity of the liquid, marked † (*viz.* 5.260 per cent NaCl). For all that, there is a great difference between both systems. For in system II, first the right minimum occurs and afterwards the left maximum; during the osmosis in system III we have just the reverse, for here the left maximum occurs first and afterwards the right minimum. Consequently, system III may indeed be represented schematically by Figs. 4 and 5, if we interchange the places of maximum and minimum with respect to one another. The scheme of this system, which the reader may easily deduce himself, contains also symbols different from those in scheme II.

Besides the paths discussed above, we have determined those of other systems:

$$\text{Water} \mid L_1' \qquad (19)$$

In Table IV the composition of the liquid L_1' of eight systems is found. The complete tables of the systems 1 (I), 4 (II) and 6 (III) are found in Tables I, II and III respectively; if any one should be interested in the other tables, I shall be pleased to forward them. The form of the path has been indicated under "type"; I means a form as in system I, etc. As in II and III, the systems 7 and 8 have a maximum in their left side path and a minimum in their right side path; as they occur, however, almost at the same time, it is difficult to say whether they belong to II or to III.

In these systems we have used two membranes H and W (no attention should be paid to the small letters a, b, etc. in Table IV): both consisted of a piece of pig's bladder; H, however, was of another bladder than bladder VI. It appears from Table IV that the paths of the eight systems (19) belong to three different types; consequently we put the question: what is the cause?

Of course the form of the path depends on the composition of liquid L_1', consequently on the position of point $1'$ in the diagram. However it appears from the table that L_1' has almost the same composition in the systems 1 and 4: yet the type of both paths is different. We see the same thing for the systems 3 and 6 and also for 3 and 7. We have already seen that the form of the path depends on $n_1:n_1'$; this influence, however, has been eliminated here as much as possible.

As has been discussed above, the form of the path, however, depends also on the membrane. It now appears from Table IV that type I occurs in all systems (*viz.* 1–3) in which bladder H has been used and type II or III in all systems in which bladder VI has been used. Consequently this makes it probable that the cause of this phenomenon must be looked for in a difference between the bladders H and VI.

This shows that the nature of the membrane influences the form of the path; later on we shall see this confirmed by other examples. If we bear in mind that all sorts of influences (*e.g.* the action of the diffusing substances, age, hysteresis, etc.) may modify the nature of the membrane (permeability, adsorptive capacity, etc.) it becomes clear that, even when using apparently the same membrane, the type of the osmosis path may differ.

The question then arises in which direction do these substances X, Y and W diffuse through the membrane? Although we shall not discuss this question until a later communication I wish to emphasize the point that great care must be used. Let us take *e.g.* scheme II or Table II which obtains for system II. We see from them that the Y amount of the left side liquid increases perpetually whereas that of the right side liquid decreases. If we should deduce from this that during the osmosis Y (Na_2CO_3) diffuses perpetually towards the left, our conclusion would not be based on a single ground; it may be wrong as well as right.

This appears at once when we consider *e.g.* the change of the X

amount on part 5.6 of the path. We see that this now increases on both sides of the membrane. For the change in concentration of a substance not only depends on the direction in which this substance passes through the membrane, but also on the directions of the other substances. The X and Y amounts of the liquids change when nothing but water diffuses. (*Cf. e.g.* normal and abnormal changes in scheme 24 of Paper I.) Consequently from the vertical arrows no conclusion may be drawn with respect to the directions in which the substances pass through the membrane. As we shall see later on, we can only deduce them by considering the synchronous changes in concentration of the three substances.

THE EFFECT OF VALENCE OF IONS ON CELLULAR PERMEABILITY TO WATER.

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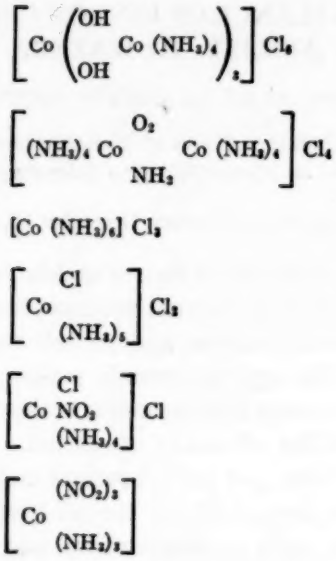
When a cell, such as the egg of the sea urchin, is placed in a solution hypotonic with respect to its natural medium, water enters under the driving force of osmotic pressure, and the cell swells. In the case of the spherical sea urchin egg the osmotic swelling proceeds relatively slowly and permits accurate measurements of the diameter, from which volume and surface of the cell can be calculated. The rate of entrance of water, per unit of time, per unit of surface and per unit of pressure may be defined as the permeability of the cell to water, and depends on a number of factors such as temperature and composition of the medium (1, 2).

While studying the effect of electrolytes on permeability of *Arbacia* eggs to water, striking differences were found in the effects of the chlorides of Ca and Mg on the one hand, and those of Na, K, Li and NH_4 on the other (2).* In quite low concentration, chlorides of the bivalent cations decreased permeability to water while those of the univalent cations had the opposite effect. Since we were unable to find constant differences in the effects of cations of the same valence, it seemed likely that we were dealing with conditions in which effect of valence predominated over other ionic effects.

If this were the case, cations of valence greater than 2 should prove still more effective in reducing permeability to water. In fact preliminary experiments with $LaCl_3$ and $ThCl_4$ gave results pointing in this direction, but solutions of these salts were so acid that we regarded them as unsuitable and looked for a valence series of cations the salts of which would form neutral solutions.

* Experiments dealing with Li and NH_4 are unpublished.

This requirement was met by a series of cobaltammine chlorides having the following structure:[1]



This series has the advantages of containing cations of widely different valence ranging from 6 to 0; the compounds form approximately neutral solutions, and have been reported to undergo complete electrolytic dissociation as determined by conductivity measurements and other methods (3).* The action of this cation series on permeability to water was studied by the following method.

Method.

Unfertilized eggs of *Arbacia punctulata* were washed in a solution of dextrose isotonic with sea water to eliminate electrolytes from the medium. The cells were then placed in a dish containing about 20 cc.

[1] The cobaltammine chlorides were kindly prepared for us by Professor Thomas P. McCutcheon, of the University of Pennsylvania. These compounds are representative of a larger number used in the experiments.

* When *Arbacia* eggs were exposed in isotonic dextrose to concentrations of these salts such as were used in the experiments most of them remained capable of fertilization and development.

of 0.38 molal dextrose solution in which had been dissolved the salt to be tested. This solution is isotonic with 40 per cent sea water (40 parts of sea water and 60 parts of distilled water). The course of swelling at constant temperature (12 ± 0.5°C.) was then observed through the microscope for 4 to 6 minutes and the mean volumes of 5 to 10 cells were plotted against time. The rate of entrance of water in cubic micra per minute is given by the rate of increase in cell volume, $\frac{dV}{dt}$; it is obtained from the slope of the curve at a given time, t. The permeability at this time is then $\frac{dV}{dt}/SP$, where S is the surface of the cell, and P is the difference in osmotic pressure between the interior of the cell at time t, and the medium.[2]

Effect of Valence of the Cation.

In the first group of experiments permeability was determined with a series of cobaltammine chlorides in 0.0001 molar concentration dissolved in hypotonic dextrose solution; the range of valence of the cation was from 1 to 6. It was found that salts with bivalent cations produced lower permeability than did those with univalent cations. But by this method, no significant difference in effect was found between the bivalent salts and those of higher valence.

The reason for this appears to be that, at a given temperature, permeability can be reduced only to a certain value. This numerical value is obtained in sea water (presumably on account of the presence of Ca and Mg) and also in dextrose solution containing cobaltammines of bivalent or higher valence in sufficient concentration.

It occurred to us that differences in effect of cobalts of higher valence might be demonstrated if we added some substance which increased permeability, and then determined the amount of cobalt necessary to reduce permeability to that obtained in sea water. This proved to be the case. It had been found that small amounts of K_2SO_4 and especially of K_3 citrate greatly increased permeability. When one of these

[2] For details of calculation see (2). In the present experiments slopes of curves were obtained by drawing tangents. Permeability varies but little during the course of an experiment; as a matter of convenience permeability was calculated at the 3rd minute.

TABLE I.

The effect of cobaltammine chlorides, having cations of different valence, on permeability to water. These salts in the concentrations shown are dissolved in 0.38 molal dextrose solution containing 0.01 M K_3 citrate. Solutions are isosmotic with 40 per cent sea water. Cell volumes are given in cubic micra $\times 10^2$. Each number represents the mean volume of 5 to 10 cells.

In the bottom row is given the permeability, which is the number of cubic micra of water entering the cell per minute, per square micron of surface, per atmosphere of pressure. The temperature was 12 ± 0.5°C.

It is seen that the effectiveness of the cobaltammine chlorides in lowering permeability increases with the valence of the cation.

The mean volume of 20 control cells in sea water was 2000×10^2 cubic micra.

Molar concentration	6 valent		4 valent			3 valent			2 valent
	1×10^{-4}	2×10^{-4}	2×10^{-4}	4×10^{-4}	8×10^{-4}	4×10^{-4}	8×10^{-4}	1.6×10^{-3}	1.6×10^{-3}
min.									
1	2156	2139	2105	2205	2200	2187	2197	2139	2191
2	2308	2253	2280	2344	2313	2363	2313	2285	2423
3	2447	2326	2447	2442	2381	2636	2466	2377	2616
4	2631	2404	2611	2591	2452	2893	2626	2490	2834
5		2533		2721	2572		2778	2601	
Permeability	0.089	0.041	0.097	0.072	0.048	0.159	0.088	0.063	0.131

TABLE II.

The effect of valence in a cation series of cobaltammine chlorides. The salts in the concentrations shown are dissolved in 0.38 molal dextrose solution containing 0.005 M K_3 citrate. Numbers represent permeability to water.

The effectiveness of the cation in reducing permeability to water increases with the valence; for example, 5×10^{-5} of the 6 valent salt are required to reduce permeability to the value usually obtained in sea water, while 64 times this concentration is required in the case of the 2 valent cobalt (data are graphed in Fig. 2).

Molar concentration	2.5×10^{-4}	5×10^{-4}	1×10^{-4}	2×10^{-4}	4×10^{-4}	8×10^{-4}	1.6×10^{-3}	3.2×10^{-3}
6 valent	0.194	0.048	0.042					
4 valent		0.172	0.058	0.038				
3 valent			0.180	0.063	0.061			
2 valent					0.171	0.113	0.157	0.043
1 valent								0.058

salts in proper concentration was added to the solution it was found that much less of 6 valent cobalt was required to reduce permeability than of the 4 valent, and so on down the series. Thus in Table I it is seen that when 0.01 M K_3 citrate was used, 0.0002 M 6 valent cobalt was sufficient to reduce permeability to the value obtained in sea water,* 0.0008 M of the 4 valent was required, while twice this amount of the 3 valent and 2 valent salts were incompletely effective.

When a lower concentration of K_3 citrate (0.005 M) was used, smaller amounts of the cobalts were necessary to reduce permeability. In Table II it is seen that 0.00005 M of the 6 valent salt was sufficient to reduce permeability, more than twice as much of the 4 valent cobalt was required, more than 8 times as much 3 valent and 64 times as much of the 2 valent salt, while this amount of the 1 valent salt was incompletely effective.

Definite though less striking differences in effectiveness were found between several bivalent and univalent cobaltammines, the former decreasing permeability to water, whereas univalent cobaltammines, as well as the undissociated compound, did not decisively either increase or decrease permeability.[3]

From these and similar experiments it is concluded that there are distinct differences in the effectiveness of cobaltammines of different valence in reducing permeability and that this effectiveness increases rapidly with the valence of the cation.

Effect of Valence of the Anion.

The results obtained with the cobalt cation series suggested the question, do anions act in the opposite direction? Do they tend to increase permeability, and is their effectiveness related to valence?

Preliminary experiments indicated that this is the case. When 0.0001 M KCl, K_2SO_4, K_3 citrate and $K_4Fe(CN)_6$ were added to dextrose solution, sulfate gave higher permeability than chloride, while citrate and ferrocyanide gave higher permeability than did sulfate.

* In 40 per cent sea water, or in 0.38 molal dextrose solution containing 0.0005 M $CaCl_2$, the value for permeability at 12°C. is approximately 0.05, but varies somewhat in different lots of cells.

[3] When cobaltammine appears as a univalent anion, $\left[\begin{matrix}\mathrm{Co} & (NO_2)_4 \\ & (NH_3)_2\end{matrix}\right]$ Na. definite increase in permeability was demonstrable.

However with such solutions, tendency to cytolysis was a disturbing factor; this was overcome by addition of $CaCl_2$ in 0.0005 molar concentration. It was now possible to determine the minimal concentration of the potassium salt which would increase permeability above the magnitude obtained in sea water or in $CaCl_2$ solution. Two series of potassium salts were used, one with inorganic, one with organic anions.

TABLE III.

The effect of valence in an anion series of inorganic potassium salts. The salts in the concentrations shown are dissolved in 0.38 molal dextrose solution containing 0.0005 M $CaCl_2$. Cell volumes measured at minute intervals are given in cubic micra $\times 10^2$. Each number represents the mean volume of 6 to 8 cells.

In the bottom row is given the permeability in the units stated in Table I. The temperature was $12 \pm 0.5°C$.

It is seen that the effectiveness of the salts in raising permeability above the value of the $CaCl_2$ control increases with the valence of the anion.

The mean volume of 20 control cells in sea water was 2236 $\times 10^2$ cubic micra.

Molar concentration	$K_4Fe(CN)_6$	$K_3Fe(CN)_6$			K_2SO_4		KCl		$CaCl_2$
	1×10^{-4}	1×10^{-4}	2×10^{-4}	4×10^{-4}	2×10^{-4}	4×10^{-4}	4×10^{-4}	8×10^{-4}	
min.									
1	2428	2423	2418	2405	2405	2433	2414	2368	2428
2	2616	2523		2626	2499	2676	2494	2509	2567
3	2861	2656	2676	2824	2616	2882	2567	2641	2671
4	3059	2757	2840	3032	2742	3154	2676	2793	2742
5	3290	2871	3026		2876		2783	2935	2824
6	3478	3005	3199		3005		2887	3059	2924
Permeability	0.118	0.057	0.081	0.115	0.062	0.133	0.046	0.073	0.048

With the former, Table III illustrating a typical experiment, shows that ferrocyanide was more effective in increasing permeability than 8 times the amount of chloride and had approximately the same effect as 4 times the amount of sulfate, while sulfate was more than twice as effective as the chloride.

The question might be raised, however, whether this result could be due to differences in concentration of potassium ions rather than to valence of the anion. That this is probably not the correct or at least the complete explanation is evident from the ratios of the effective concentrations as given in the preceding paragraph.

Table IV brings out the effect of valence still more clearly. In this experiment 0.001 M ferrocyanide was required definitely to increase permeability above the value of the control ($CaCl_2$ solution), twice as much ferricyanide, 4 times as much sulfate and 8 times as much chloride.

TABLE IV.

The effect of valence in an anion series of inorganic potassium salts. The salts in the concentrations shown are dissolved in 0.38 molal dextrose solution containing 0.0005 M $CaCl_2$. Numbers represent permeability to water.

The effectiveness of the salts in increasing permeability above the value of the $CaCl_2$ control increases with valence of the anion.

Molar concentration	2×10^{-4}	8×10^{-4}	1×10^{-3}	2×10^{-3}	4×10^{-3}	8×10^{-3}
KCl				0.066	0.067	0.095
K_2SO_4				0.048	0.152	
$K_3Fe(CN)_6$			0.068	0.107		
$K_4Fe(CN)_6$	0.056	0.063	0.087	0.192		

($CaCl_2$ = 0.046)

TABLE V.

The effect of valence in an anion series of organic potassium salts.

The effectiveness of the salts in increasing permeability above the value of the $CaCl_2$ control increases rapidly with the valence of the anion (data are graphed in Fig. 1).

Molar concentration	2.5×10^{-4}	5×10^{-4}	1×10^{-3}	2×10^{-3}	4×10^{-3}	8×10^{-3}
K acetate			0.044	0.049	0.048	0.116
K_2 tartrate		0.055	0.060	0.090	0.150	
K_3 citrate	0.052	0.086	0.184			

($CaCl_2$ = 0.046)

Similar results were obtained using a series of organic salts with increasing valence of the anion (Table V): 0.0005 molar citrate was required to increase permeability above the value of the $CaCl_2$ control; 4 times as much tartrate and 16 times as much acetate were needed.

In both inorganic and organic anion series it was invariably found that the effectiveness of the salts in increasing permeability to water increases rapidly with the valence of the anion.

The relation between permeability and valence in the anion and cation series is graphically shown in Figs. 1 and 2. In the first graph (Fig. 1) permeability to water is plotted against the molar concentration of an anion series. It is seen that with the trivalent anion a relatively slight increase in concentration causes a marked change in

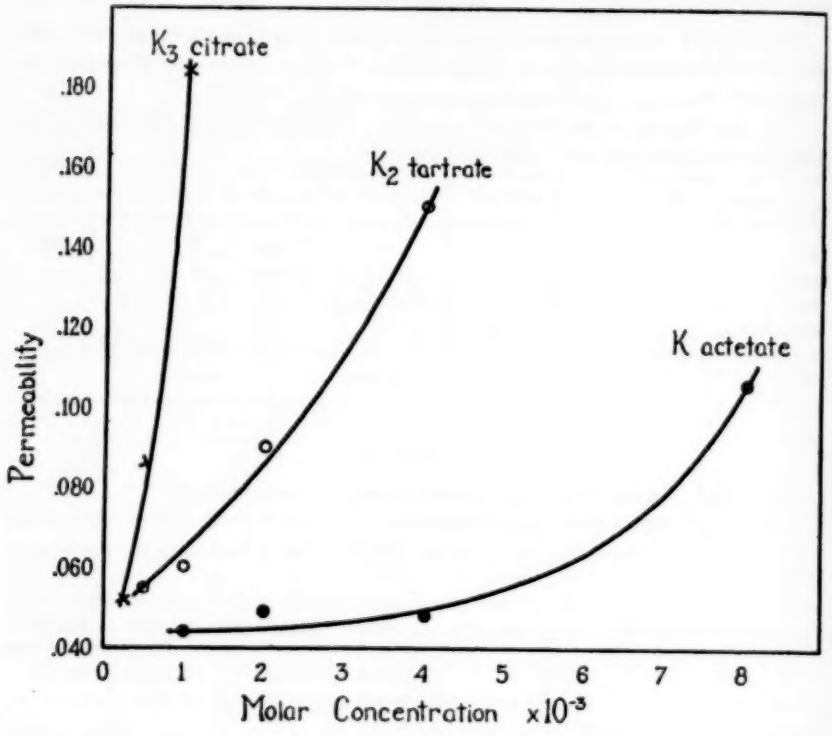


Fig. 1. The relation between permeability and molar concentration of salts having anions of different valence (data are taken from Table V).

permeability; whereas with the univalent anion a considerable change in concentration is required to bring about an increase in permeability; and the bivalent anion occupies an intermediate position.

Similarly a plot of a cation series (Fig. 2) shows that permeability to water decreases with concentration of the 6 valent cation more rapidly than with cations of lower valence.

DISCUSSION.

The experiments indicate that permeability of cells to water is regulated, at least in part, by the sign and the number of charges on the ions in the medium, in the sense that cations decrease and anions increase permeability to water; and these effects increase rapidly with the valence of the ion.

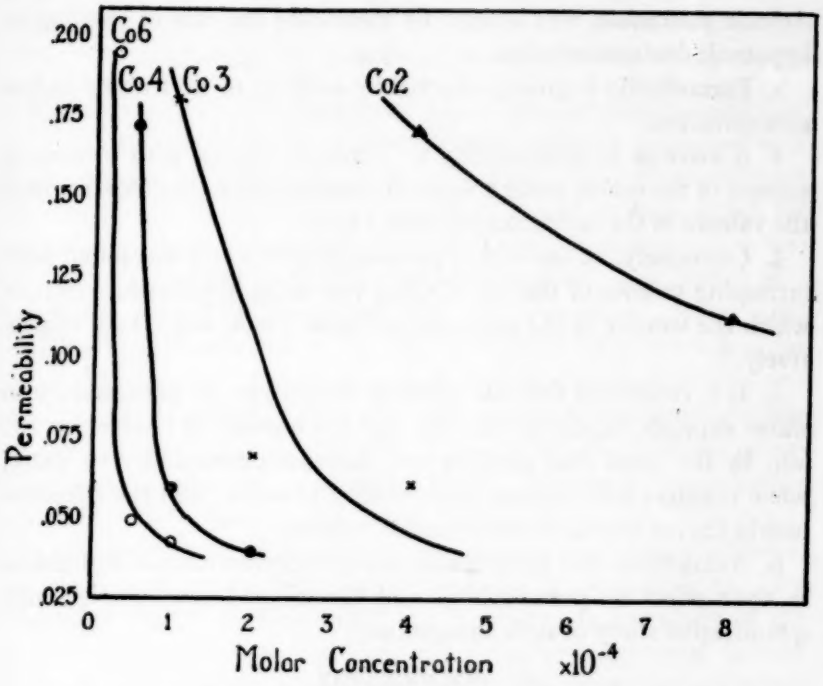


Fig. 2. The relation between permeability and molar concentration of salts having cations of different valence (data are taken from Table II).

It is interesting to note that valence and the sign of the charge were found by Osterhout (4) and Raber (5) to produce changes in conductivity of plant tissues that are closely paralleled in our experiments on permeability to water.

The experiments also show that it is possible to determine the concentration of a cation which will oppose the effect of an anion in its tendency to increase permeability to water, and conversely, the

restraining action of a cation on osmosis may be balanced by the proper concentration of an anion. The method used, therefore permits quantitative study of antagonism between cations and anions in their effects on permeability to water.

SUMMARY.

1. Permeability to water in unfertilized eggs of the sea urchin, *Arbacia punctulata*, was studied by measuring the rate of swelling in hypotonic dextrose solution.

2. Permeability is greatly affected by addition of electrolytes in low concentration.

3. A *decrease* in permeability to water was found with increasing valence of the *cation*, using a series of cobaltammine chlorides in which the valence of the cation ranged from 1 to 6.

4. Conversely, an *increase* in permeability to water was found with increasing valence of the *anion*, using two series of potassium salts in which the valence of the anion ranged from 1 to 4, and 1 to 3, respectively.

5. It is concluded that the effect of electrolytes on permeability to water depends chiefly on the sign and the number of charges on the ion, in the sense that positive ions decrease permeability to water, while negative ions increase permeability to water; and the effectiveness of the ion is greater the higher its valence.

6. Antagonism has been demonstrated between cations and anions in their effect on permeability, and the method employed permits quantitative study of such antagonism.

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ON PYRIDINE HEMOCHROMOGEN.

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The writers (1, 2) have shown that every hemochromogen consists of reduced heme (the iron pyrrol complex of hemoglobin) combined with some nitrogenous substance. They have furthermore given evidence (2) that in cyanide hemochromogen there is one cyanide group for every heme group. There had previously been two attempts to determine the composition of pyridine hemochromogen. Von Zeynek (5), before the nature of hemochromogen was understood, found solid pyridine hemochromogen to contain 2.2 molecules of pyridine per heme. He regarded his single experiment as only preliminary and gave no certain evidence that the solid analyzed was pure pyridine hemochromogen. Recently R. Hill (3) concluded that pyridine hemochromogen contains two pyridines per heme. This conclusion is hardly justified by Hill's experiments. And Hill did not consider two important difficulties, one concerned with the precipitation of reduced heme, the other with the aggregation of pyridine hemochromogen. We propose to discuss in some detail the difficulties in the study of the pyridine hemochromogen equilibrium for they are of general interest in the chemistry of the hemochromogens. The complications of the pyridine reactions will be easier to understand if we first review the simpler reactions with cyanide.

The Reactions between Cyanide and Reduced Heme.

One molecule of KCN per molecule of reduced heme and not less suffices to convert a 2×10^{-4} M solution of reduced heme into cyan-hemochromogen. This proves that cyan-hemochromogen does not contain more than one cyanide group per heme. It does not exclude the possibility that cyan-hemochromogen contains less than one cy-

anide group per heme. For we have shown (1,2) that in every hemochromogen there is the equilibrium:

$$\text{Hemochromogen} \rightleftarrows \text{Nitrogenous substance} + \text{reduced heme.}$$

So some of the cyanide added must be free to drive the equilibrium

$$\text{Cyan-hemochromogen} \rightleftarrows \text{Cyanide} + \text{reduced heme}$$

to the left. The following considerations, however, indicate that the free cyanide in the 2×10^{-4} M solution of cyan-hemochromogen is negligibly small.

In a mixture of reduced heme and hemochromogen the fraction of the total pigment which has to be in the form of reduced heme in order for the spectrum of reduced heme to be seen is independent of the total pigment concentration provided the more dilute the solution the greater the thickness of it through which one looks. Let us assume that the concentration of free cyanide needed to prevent the appearance of reduced heme, that is, to convert a given fraction of the pigment into hemochromogen, is independent of the total pigment concentration in concentrations less than 2×10^{-4} M. On this hypothesis as the total concentration of pigment is made smaller, the bound cyanide becomes smaller while the free cyanide remains the same. In a very dilute solution, then, the free cyanide is approximately equal to the total cyanide added. We found that in such a dilute solution it takes 3.75×10^{-5} M cyanide to prevent the appearance of reduced heme. If the free cyanide is likewise 3.75×10^{-5} M in the relatively concentrated 2×10^{-4} M solution where it takes only 1 molecule of KCN to convert a molecule of reduced heme into hemochromogen, then in this case the free cyanide is within the experimental error negligible and so cyan-hemochromogen probably does not contain less than one cyanide group per heme.

The Reactions between Pyridine and Reduced Heme.

In his study of the composition of hemochromogen R. Hill (3) used pyridine as his nitrogenous substance. We had pointed out (1) that of the substances originally tested pyridine and nicotine had the greatest affinity for reduced heme. Later experiments, however, showed that cyanide has an affinity for reduced heme some fifty times

greater than that of pyridine. Hence the use of cyanide in the experiments just described and the avoidance so far as possible of some of the serious difficulties which will now be discussed.

Hill determined the number of pyridine molecules per heme needed to prevent the appearance of reduced heme in solutions of various concentrations. He states that reduced heme can be detected if present as 5 per cent of the total pigment. Columns 1 to 3 of Table I give Hill's results. The other columns give the calculations omitted by Hill which we based on his results.

In the most dilute solution (0.62×10^{-3} M) the total pyridine was 2.34×10^{-3} M. If one pyridine was bound per heme the free pyridine was $(2.34-0.62)\times10^{-3}$ M or 1.72×10^{-3} M. If two pyridines were

TABLE I.

Total heme M × 10³	Total pyridine M × 10³	Total pyridine / Total heme	Free pyridine = 1.72 × 10⁻³ M		Free pyridine = 1.10 × 10⁻³ M	
			Bound pyridine M × 10³	Pyridine bound per heme	Bound pyridine M × 10³	Pyridine bound per heme
7.69	14.6	1.9	12.9	1.68	13.5	1.76
3.42	6.50	1.9	4.78	1.40	5.40	1.58
2.20	4.62	2.1	2.90	1.32	3.52	1.60
1.62	3.56	2.2	1.84	1.14	2.46	1.52
1.18	2.83	2.4	1.11	.94	1.73	1.46
.62	2.34	3.8	.62	1.00	1.24	2.00

bound the free pyridine was $(2.34-2\times0.62)\times10^{-3}$ M or 1.10×10^{-3} M. Let us *assume*, since some assumption is necessary, that the free pyridine was the same in all the solutions. The bound pyridine in the more concentrated solutions may now be obtained by subtracting the hypothetically constant free pyridine from the total pyridine added. Finally the values of pyridine bound per heme are calculated by dividing the bound pyridine by the total heme. These values are inconsistent and all less than two.

Hill's conclusion that pyridine hemochromogen contains two pyridine groups per heme thus rests on the unproven assumption that the free pyridine was negligible in his most concentrated solutions. If the free pyridine was the same in all the solutions then this assumption is incorrect. Furthermore, we shall now present evidence which indi-

cates that the free pyridine in the concentrated solution was actually greater than in the dilute solutions, that the corrections for the free pyridine should be greater than they are in Table I rather than less as is necessary to justify Hill's conclusion.

The Precipitation of Reduced Heme.—Cyanide has an affinity for reduced heme some fifty times greater than that of pyridine. In order, therefore, for the free nitrogenous substance to be negligible compared with the bound a solution of pyridine hemochromogen must be some fifty times as concentrated as a similar solution of cyan-hemochromogen. Hill's solutions, it has been pointed out, were not concentrated enough for the free pyridine to be negligible. But they were concentrated enough to introduce the problem of the precipitation of reduced heme.

Let us suppose that in the equilibrium mixture

$$\text{Hemochromogen} \rightleftarrows \text{Nitrogenous substance} + \text{reduced heme}$$

90 per cent of the pigment is in the form of hemochromogen. If the total concentration of pigment is low enough the 10 per cent of reduced heme formed on adding the reducing agent will remain in solution. This small percentage of reduced heme cannot be detected spectroscopically. If, however, the total concentration of pigment is high enough, the amount of the sparsely soluble reduced heme formed will be greater than the amount which can stay in solution. Reduced heme will precipitate, more hemochromogen will dissociate to maintain the equilibrium and so on. Under suitable conditions the continued formation of reduced heme can readily be followed with the spectroscope. Finally, the concentration of reduced heme in solution will correspond to the solubility of reduced heme or at least to some condition where reduced heme does not precipitate rapidly. But the total reduced heme in solution and a precipitate will now be more than 10 per cent of the total pigment and the spectrum of reduced heme will be seen.

It follows from these considerations that to prevent the precipitation and hence the appearance of reduced heme, it is sufficient in the dilute solution to convert 90 per cent of the pigment into hemochromogen leaving 10 per cent as reduced heme. In the concentrated solution, however, one cannot have in solution as reduced heme so much as 10

per cent of the total pigment and so more than 90 per cent must be converted into hemochromogen. This can be done only by increasing the concentration of free nitrogenous compound. To prevent, therefore, the appearance of reduced heme one must have a higher concentration of free nitrogenous compound in the concentrated solution than in the dilute solution.

As shown in Table I 1.9 pyridine molecules per heme have to be added to a 7.69×10^{-3} M solution of reduced heme in order to cause the disappearance of the reduced heme spectrum. If the free pyridine here is 1.72×10^{-3} M as in the more dilute solution, then 1.68 pyridines are bound per heme. But in the concentrated solution of pyridine hemochromogen the difficulty concerning the precipitation of reduced heme enters in to an unknown extent. The free pyridine needed to prevent the appearance of reduced heme would on this basis be higher than the 1.72×10^{-3} M needed in the dilute solution and fewer than 1.68 pyridines would be bound per heme. Hill did not take this difficulty into account.

The Aggregation of Pyridine Hemochromogen.—The second difficulty in the study of the pyridine hemochromogen equilibrium is concerned with the possible aggregation of the hemochromogen itself. Pyridine hemochromogen in a 10 per cent pyridine solution has a typical sharp narrow hemochromogen α band, which is about 25 Å. u. toward the blue of the α band of globin hemochromogen. If the solution contains only 0.004 M pyridine and just enough pigment so that one can readily see the hemochromogen band when looking through a layer 2 cc. thick, then immediately on reduction the α band is in the same position as in the 10 per cent pyridine solution. But within a few seconds there is a shift of the band toward the red of about 30 Å. u. and within a few minutes a further shift of about 10 Å. u. The band, furthermore, becomes broader and asymmetrical, the part toward the red being darker than the part toward the blue. On half saturation with $(NH_4)_2SO_4$ the band shifts still further toward the red and becomes still more asymmetrical. All this is in harmony with our earlier observations (1) and with those of Keilin (4).

In our original paper (1) we showed that such shifts in the position of a hemochromogen band are usually accompanied by a precipitation of the hemochromogen. Keilin (4) has suggested that it is the change

in the dispersion of the pigment to which the change in the spectrum is directly due. This correlation between dispersion and spectrum is most easily observed with globin hemochromogen. If this hemochromogen is precipitated by salting out in alkaline solution, by neutralization, or by dilution of a neutral concentrated urea solution then the band shifts some 25 Å. u. toward the red.

The observations on pyridine hemochromogen therefore indicate that when the solution is dilute in respect to pyridine the hemochromogen is not molecularly dispersed. Hill did not recognize this difficulty nor is it clear how this difficulty even when recognized can be taken into account in the calculations.

CONCLUSIONS.

1. Cyanide hemochromogen probably contains one cyanide group per heme group.

2. The equilibrium between pyridine hemochromogen and its components, pyridine and reduced heme, is complicated to an unknown extent by the precipitation of reduced heme and the aggregation of pyridine hemochromogen.

3. These complications were not taken into account in R. Hill's experiments on pyridine hemochromogen.

4. Even if Hill's experiments are sound they do not prove his conclusion that pyridine hemochromogen contains two pyridine groups per heme group.

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THE INFLUENCE OF SIZE, SHAPE AND CONDUCTIVITY OF MICROSCOPICALLY VISIBLE PARTICLES ON CATAPHORETIC MOBILITY.

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INTRODUCTION.

Although the cataphoresis of microscopically and submicroscopically visible particles has been very frequently investigated, the influence of size, shape and conductivity of the particle has not been completely determined. Solution of this problem is of the utmost importance for all applications of the method of cataphoresis. Before differences in mobility in an electric field may be ascribed to variations in the nature of the electric conditions at the phase boundary of the particle, the influence of variation of size, shape and conductivity of particles equal in other respects must be recognized. The theories of Helmholtz, Lamb, and von Smoluchowski (1) lead to the formula for cataphoresis.

$$V = \frac{1}{4\pi} \frac{X D \zeta}{\eta},$$

(V = cataphoretic velocity. X = field strength. D = dielectric constant of the medium. ζ = electrokinetic potential. η = viscosity of the medium. All units c.g.s. electrostatic.) According to this formula neither size nor shape should influence the migration velocity. Debye and Hückel (2), making certain assumptions, came recently to the conclusion that the factor $\frac{1}{4\pi}$ was valid only for cylindrical particles whereas the factor $\frac{1}{6\pi}$ should be substituted in the case of spherical particles. This difference is great enough to be determined experimentally.

Previous experiments dealing with the effect of size, shape and conductivity may be briefly summarized as follows. Hardy (3) found that various solutions of submicroscopic particles of protein prepared by adding different amounts of acetic acid to the protein solutions had identical cataphoretic mobilities. As the different solutions varied in diffraction color, it was believed that the mobility observed was independent of particle size. Burton (4) found that Bredig silver solutions presumably of different particle size had equal cataphoretic velocities. Ellis (5), however, referring to a statement of Cotton and Mouton that the velocity varies with size, compared droplets of the same size only. McTaggart (6) noted that gas bubbles 60μ to 160μ in diameter migrated independently of the diameter. In contrast to these findings are the experiments of Mooney (7). He found that for droplets of Nujol* 0.5μ to 40μ in diameter, there was a considerable increase in migration velocity with increasing diameters of the droplets. Thus for a 22 fold increase in diameter, from 0.5μ to 11μ, the velocity was doubled. Mooney obtained similar results for ten other kinds of emulsions made up in water and in dilute electrolytes. In $CuSO_4$ solutions these differences supposed by Mooney to depend upon particle size disappeared. The technic employed by Mooney was different from that used by most recent authors. He worked with a vertical, cylindrical, capillary glass tube with sealed in electrodes. The effect of gravity on the droplets, although presumably accounted for in estimation of velocity, was considerable. These observations of Mooney are unique in that such marked differences in velocity had never previously been described. Freundlich and Abramson (8) and Abramson (9) found that (1) single red blood cells and their aggregates of different sizes and shapes, (2) irregularly shaped polymorphonuclear leucocytes, (3) quartz particles of various sizes (0.5μ to 15μ) and shapes in water, dilute electrolytes and non-electrolytes, (4) glass and clay particles suspended in similar solutions, all migrated independently of size and shape. McTaggart's experiments with air bubbles were also confirmed by studying air bubbles in gelatin gels.

Although there is no extensive theoretical treatment of the influence of particle conductivity on cataphoretic mobility, the theories have always been derived for insulators.

* A paraffin oil.

In connection with the influence of the conductivity of the particles two problems arise for discussion. First, the whole particle may be homogeneous. In this case the influence of the conductivity of the particle cannot be investigated experimentally as the conductivity of the particle cannot be varied without varying the composition of the whole particle including its phase boundary. Secondly, the particle may be heterogeneous when the surface film is composed of material entirely different from that of the bulk. Particles having identical surface films, but varying in the conductivity and chemical make-up of the enclosed bulk of the particles may be then compared. Such particles can be obtained by suspending particles of varied chemical nature and electric conductivity in dilute protein solutions (10). The adsorbed film of protein determines the properties of the surface which is responsible for the mobility of the particle, while the chief material is excluded from direct contact with the medium. If the material making up the bulk of the particle were to influence the velocity of migration, then this influence could only be due to the varying conductivity if the influence of size and shape of particle can be excluded.

Methods.

Apparatus.—A horizontally fixed microscopic cataphoresis cell similar in arrangement to that described by Northrop and Kunitz (11) was employed. Certain modifications were found useful which have been described in detail elsewhere (12). The cell was of Pyrex glass, about 0.8 mm. in depth, 9 mm. wide, and the hydrodynamic conditions followed the principles laid down for such systems by von Smoluchowski (1). Non-polarizable electrodes were used. The applied E.M.F.'s were great enough to give easily measurable velocities with no heating effects.

Materials.—Droplets of Nujol, benzyl alcohol, castor oil, a paraffin oil, and coca butter were studied in media (alcohol-water, cane sugar-water mixtures containing traces of electrolytes) of approximately the same specific gravity as the oils themselves. Certain droplets were studied in systems containing sufficient protein to give the droplets at least a complete surface of protein. This procedure gives very uniform surfaces because of the similarly adsorbed protein films, and was

TABLE I.

Nujol in Water-Ethyl Alcohol Mixture Having Same Density as the Oil. A Few Drops of pH 7.4 Phosphate Buffer Added to About 50 cc. of Emlsion. The Same Result Was Obtained for Emulsions in Methyl Alcohol Mixtures.

Diameter of droplet	Time to move 200μ	Diameter of droplet	Time to move 200μ
μ	sec.	μ	sec.
30	15.2	0.5	14.5
1	15.3	13	14.5
5	15.2	13	14.6
3	14.6	5	14.4
13	14.7	1	14.4

TABLE II.

Benzyl Alcohol + Small Amount of Sugar + Few Drops pH 7.4 Buffer.

Diameter of droplet	Time to move 300μ	Diameter of droplet	Time to move 300μ
μ	sec.	μ	sec.
45	13.5	20	14.0
5	14.2	20	13.8
2	14.2	2	14.4
25	14.2	15	14.0
2	14.2	3	13.8
1	14.0	1	13.6

TABLE III.

Paraffin Oil in Ethyl Alcohol-Water Mixture + Few Drops pH 7.4 Phosphate Buffer.

Diameter of droplet	Time to move 100μ	Diameter of droplet	Time to move 100μ
μ	sec.	μ	sec.
25	9.8	20	8.4
2	9.0	2	8.2
5	8.8		
20	9.4	2	6.0
		15	6.2
15	12.0		
3	12.2		

The brackets are groups from different levels in the cataphoresis cell.

TABLE IV.

Coca Butter.

Experiment (*a*). In distilled water without protein film			*Experiment* (*b*). The suspension was here in 0.05 per cent gelatin + dilute HCl (about N/1000)		
Diameter of droplet	Time to move same distance	Remarks	Diameter of droplet	Time to move same distance	Remarks
μ	sec.		μ	sec.	
8	5.2	Note a greater variation than in part (*b*)	8	4.6	The speed of the droplets is more uniform and independent of the size with the protein film
5	5.4		1	4.2	
3	6.2		4	4.4	
2	6.0		4	4.6	
2	6.0		2	4.6	
4	5.2		7	4.4	
7	5.4		5	4.6	
2	5.6		1	4.4	
			5	4.4	
			1	4.4	

TABLE V.

Castor Oil in Distilled Water + Few Drops of pH 7.4 Phosphate Buffer.

Diameter of droplet	Time to move 200μ	Diameter of droplet	Time to move 200μ
μ	sec.	μ	sec.
2	5.0	1	5.4
3	5.2	7	5.2
2	5.0	1	5.4
1	5.2	10	5.5
1	5.4	5	5.5

therefore used to study needles and droplets simultaneously. Asbestos needles of various lengths remained fairly well suspended in watery and dilute protein solutions, as did recrystallized *m*-aminobenzoic acid crystalline needles. In all experiments the following precautions were observed:

1. The vertical movements of the particles due to gravity were eliminated as far as possible, by having the density of the medium and of the particle similar.

2. Measurements were always made at the stationary water layer in the cell to make sure that the mobility observed in the mid-regions

(where most observations reported have been made) was not simply due to electroendosmotic streaming of the water within the cell.

EXPERIMENTAL.

1. The Effect of Particle Diameter on Cataphoretic Mobility.—Droplets of the following substances were studied: The details are in the tables where typical protocols are presented.

Nujol 0.5 μ to 35 μ Table I.
Benzyl alcohol 0.5μ to 45μ Table II.
Paraffin oil 2μ to 25μ Table III.
Coca butter 1μ to 8μ Table IV.
Castor oil 1μ to 10μ Table V.

All our experiments have been performed in the presence of sufficient electrolyte or protein to avoid the complications found in Mooney's experiments. As mentioned previously, this author noted that in electrolyte-poor emulsions, droplets having larger diameters move more rapidly than smaller droplets. Mooney also noted that this difference in velocity was less in the presence of electrolytes. The observations of Mooney have been confirmed by the authors in very electrolyte-poor emulsions containing no proteins on a few occasions.

An analysis of the five tables just mentioned discloses that under the stated conditions *the cataphoretic velocity of oil droplets is independent of their diameter.* The larger drops were easily studied and as has been described under methods, media of almost identical specific gravity with that of the droplets were used. Very rarely (less than about $\frac{1}{5}$ of 1 per cent of all observations) anomalous behavior of droplets was observed. A droplet would migrate very quickly or very slowly. The relatively large changes produced by small amounts of impurities in the system is sufficient to account for these variations which are in no way related to their size or shape.

2. The Effect of Particle Length on Cataphoretic Velocity.—Asbestos pulp in dilute suspensions gives needles of easily studied lengths from 3μ to 200μ. The diameter of the needles was from about 1μ to 3μ. Long cylinders of very small radius were, therefore, available for study. These needles were studied suspended in distilled water and in dilute

protein solutions. Fig. 1 gives the data for these two types of media. In the figure the orientation of the particle is given by its position, the length of the particle by the scale and the time in seconds taken for the particles to migrate a given distance, the same for all particles, by

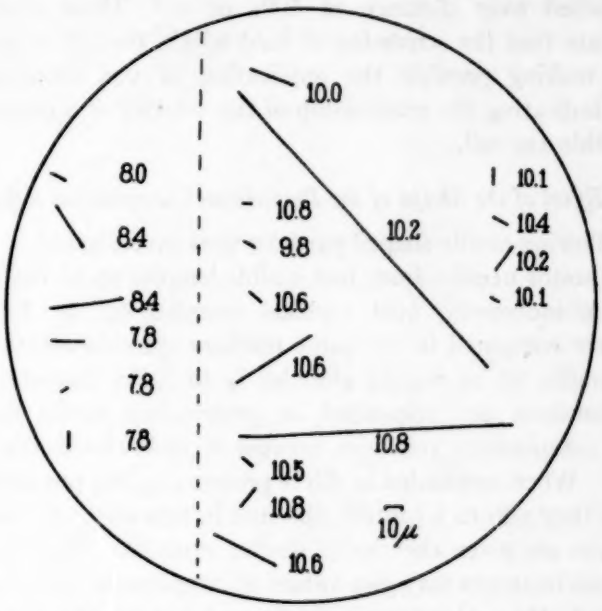

FIG. 1. The field is divided into two parts. In right lower hand corner is the scale to which the asbestos needles have been drawn. The slight differences in radius were neglected. The group of needles to the left of the dotted line were studied in distilled water with the orientation as when observed; the number to the right of each needle is the time in seconds required to migrate a given distance which was the same for the whole group. The needles to the right of the dotted line were studied in N/1000 HCl containing 0.05 per cent gelatin which coats the needles with a protein film. The numbers have the previous significance. This figure demonstrates that neither size nor orientation of particle changes the cataphoretic velocity.

the number next to the particle. It is evident that for such particles *the cataphoretic velocity is independent of particle size*, thus confirming with particles of entirely different shape the results obtained with oil droplets.

Orientation of Needle-Shaped Particles during Migration.

Most of the needle-shaped particles long enough to have no brownian movement are found in horizontal planes, but oriented at random within these planes. During the migration this random orientation is not disturbed over distance of 500μ or so.[1] These observations demonstrate that the streaming of fluid within the cell is practically laminar, making possible the application of von Smoluchowski's formula, indicating the relationship of the velocity of a particle to its depth within the cell.

The Effect of the Shape of the Particle on Cataphoretic Velocity.

The following needle-shaped particles were investigated:

(*a*) Asbestos needles from just visible lengths up to 70μ long.

(*b*) *m*-Aminobenzoic acid crystals (needles up to 100μ long). These were compared in the same medium (protein solutions) with small paraffin oil or mastix globules 1μ to 5μ in diameter. When such substances are suspended in protein-free media they have different cataphoretic velocities because of their chemically different surfaces. When suspended in dilute protein (*e.g.* 0.1 per cent gelatin) solutions they adsorb a protein film and in this way, the needles and oil globules are given chemically similar surfaces. That the protein film in such instances may give values of cataphoretic velocity remarkably close to the native protein has been shown by Abramson (9) and by Freundlich and Abramson (8). These experiments have been confirmed by Briggs (13) using an entirely different method.

Fig. 2 shows groups of particles studied at the same level. The particles are drawn to the scale indicated or are of the size noted. An oil droplet and a needle were always studied at the same level at the same time. The time of migration over a given distance is given in seconds next to the particle. These experiments demonstrate that *for very extreme instances of particle size and shape the velocity of cataphoretic migration is here determined solely by the surface characteristics of the particle and is independent of both size and shape within the limits studied.*

[1] Red cell rouleaux have also been observed to exhibit the same phenomenon in serum and other liquids. The same was true for gelatin gels (8, 9). See also Abramson, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxvi, 147.

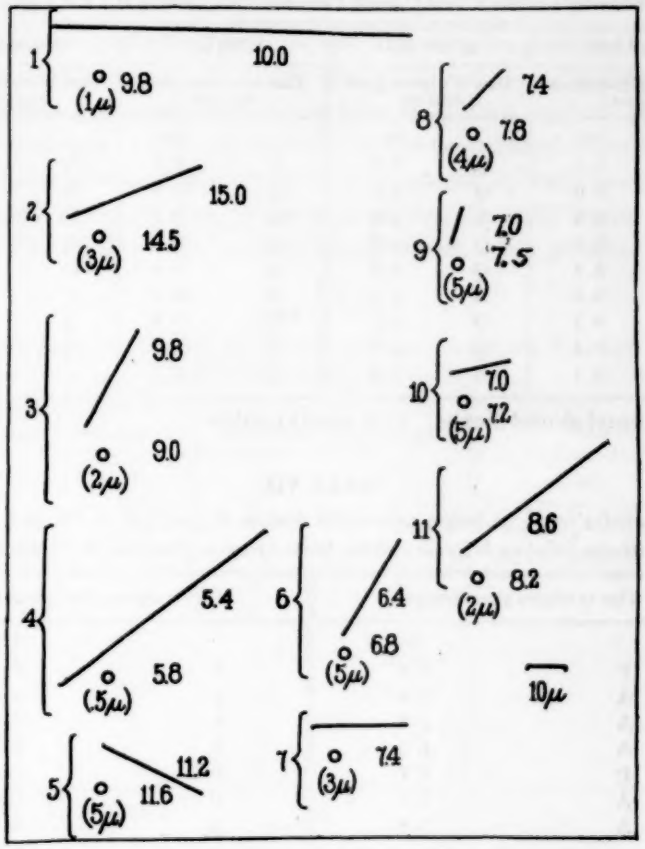

FIG. 2. The brackets indicate needles and globules studied in pairs. Typical experiments, Nos. 1–3, are *m*-aminobenzoic acid crystals and mastic globules covered with gelatin. Nos. 4–10 are asbestos needles and paraffin oil globules covered with albumin. No. 11 is a *m*-aminobenzoic acid crystal and a paraffin oil globule covered with gelatin. The oil droplets and mastic particles are all drawn the same size but their diameter is given in parentheses near the particle. The needles are drawn to the scale in the lower right hand corner.

Each pair was studied at the same level in the cell. The numbers following each particle are the relative speeds. It is evident that there is no change in cataphoretic velocity even for these limiting cases.

TABLE VI.

The Migration Velocity of Quartz and Benzyl Alcohol Droplets in Approximately N/100 and N/1000 HCl When Both Quartz and Benzyl Alcohol Have Similar Adsorbed Gelatin Films (Initial Concentration Gelatin 0.2 Per Cent).

N/100 HCl		N/1000 HCl		N/100 HCl		N/1000 HCl	
Time to migrate given distance		Time to migrate given distance		Time to migrate given distance		Time to migrate given distance	
	sec.		sec.		sec.		sec.
B	9.4	Q	5.8	B	9.5	Q	6.2
Q	9.0	Q	6.0	Q	10.2	B	6.0
Q	9.4	B	5.8	Q	9.2	B	5.8
B	9.0	Q	6.2	Q	9.4	Q	6.2
Q	8.8	Q	5.8	Q	9.4	Q	5.8
Q	9.6	B	6.0	B	10.0	Q	5.4
B	9.2	Q	5.6	Q	9.8	Q	5.8
Q	9.4	Q	6.0	Q	9.6	Q	5.5
B	9.1	B	5.8	Q	9.5		

B = benzyl alcohol droplet. Q = quartz particle.

TABLE VII.

Agar Particles in Equilibrium with N/50 Acetate Buffer, pH = 3.6, in ½ Per Cent Gelatin Solution Migrate with the Same Speed as Paraffin Oil Droplets.

Time to migrate given distance		Time to migrate given distance	
	sec.		sec.
P	6.4	A	6.4
A	6.4	A	6.2
A	6.4	A	6.0
A	6.6	P	6.2
P	6.7	P	6.0
A	7.0	P	6.0
A	6.8	A	6.8
		A	6.0

The Same Experiment with Hemocyanine, pH = 4.0

	sec.		sec.
P	7.0 }	P	7.8 }
A	7.4 }	A	7.0 }
A	6.5	A	7.0 }
A	6.4		
A	6.8	P	9.0 }
P	7.2	A	8.4 }
A	7.0		
A	7.0		

Brackets refer to droplets studied at particular levels in the cataphoresis cell. P = oil. A = agar.

The Influence of Particle Conductivity.

The following kinds of particles, all covered with protein films, were studied simultaneously in dilute electrolyte solutions: paraffin droplets, quartz particles, droplets of benzyl alcohol, carbon particles, particles of agar. The conductivity of the material of these particles varies from virtually zero in the case of quartz, up to a conductivity practically equal to that of the surrounding medium in the case of agar. The protein film was obtained either with gelatin or with

TABLE VIII.

Carbon Particles Suspended in N/100 *HCl* + ½ *Per Cent Gelatin Move with the Same Speed As Paraffin Oil Droplets.*

Time to migrate given distance		Time to migrate given distance		Remarks
	sec.		sec.	
P	6.0	P	5.8	Brackets refer to particles studied at different levels in cataphoresis cell
C	6.0	C	6.0	
P	5.8	P	9.0	
C	5.8	C	9.4	
P	4.5	P	8.8	
C	4.8	C	9.4	
P	6.6	P	5.6	
C	7.0	C	5.4	

P = oil. C = Carbon.

hemocyanine (*Limulus*). Tables VI, VII and VIII show that *the velocity of cataphoresis does not depend to any measurable extent on the bulk conductivity of the particle.*

CONCLUSION.

The electrophoretic mobility of microscopically visible particles is independent of size, shape and conductivity of the particle within the limits of the experimental error. This is valid for extreme variations in size, shape and conductivity.

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